REMARKS

Claims 1, 5-10, 12-14, 17 and 19 were pending and examined in the June 16, 2005 final Office action. In this reply, claims 1, 12, 17 and 19 have been amended for clarity. Accordingly, claims 1, 5-10, 12-14, 17 and 19 will be pending upon entry of this amendment.

Specifically, claim 12 has been amended to provide express antecedent basis for recitation of "the gp39 antagonist" in claim 13. Support is found in the specification as filed, for example at page 4, lines 7-17. Claims 17 and 19 have been amended for clarity. Support is found in the specification as filed, for example at page 6, second full paragraph citing WO 95/06666, which is incorporated by reference on page 9, lines 20-22 of the instant specification. The use of "variable" and "hypervariable" regions in the construction of chimeric and humanized antibodies, respectively, was known and is described *inter alia* on page 9, lines 1-20, of WO 95/06666 (see also, col. 8, lines 4-33 of 5,747,037, an equivalent US patent of this disclosure).

Rejection Under 35 U.S.C. § 112 - Written Description

The Examiner maintained rejection of claims 1, 5-10, 17 and 19 under 35 U.S.C. § 112 for allegedly lacking written description support for "<u>one or more</u> autoantigens" (emphasis in Office action). The Examiner reasoned that the specification at pages 9-11 and Example 1 does not adequately support the "one or more" feature as recited in the claims. To expedite prosecution without conceding correctness, claim 1 has been amended to delete reference to the "one or more" feature, thereby obviating this rejection. For the record, however, applicant respectfully maintains that the support on page 3, lines 25-36 of the specification supports recitation of "one or more" autoantigens in the context of these claims (emphasis added):

> The language "autoimmune disorder" is intended to include disorders in which the immune system of a subject reacts to **autoantigens**, such that significant tissue or cell damage occurs in the subject. The terms "**autoantigen**" and "self-antigen" are used interchangeably herein...The term "self" as used herein is intended to mean any component of a subject and includes molecules, cells, and organs. **Autoantigens** may be **peptides**, nucleic **acids**, or other biological <u>substances</u>.

It is respectfully submitted that the Examiner has misapprehended this passage, pages 9-11, and Example 1, in view of the context for "one or more" autoantigens. The Examiner's reasoning in this regard, now moot in view of the amendment, is nevertheless not understood. The present claims are *not* directed to undisclosed T cell autoantigens. Instead, the claims are directed to methods for inhibiting T-cell-mediated tissue destruction resulting from an immune reaction *against* such autoantigens. To practice the claimed methods, it is not required that one even *know* the identity of an autoantigen. According to the methods, inhibiting T cells activated by an autoantigen will prevent continued T-cell-mediated tissue destruction. Thus, the Examiner's apparent requirement for a written description of the identity of all possible autoantigens is misplaced in view of the context of these claims.

On page 3, paragraph 2, of the Office action, the Examiner further alleged a lack of written description for the phrase "wherein the anti-gp39 antibody or fragment binds to an epitope which is specifically bound by a monoclonal antibody produced by 24-31 hybridoma." The Examiner required cancellation of the alleged new matter or an indication of sufficient written description support for this phrase.

In response, it is respectfully submitted that the subgenus of 24-31 antibody epitopic specificity was described in the application as filed. The support citations of record from pages 4-6 of the specification (and the references cited therein) refer to chimeric and humanized antibody technology. At the time of the invention, once a specific monoclonal antibody such as 24-31 was in hand, a subgenus having that specificity was inherently or impliedly, if not explicitly, described, given this well known technology. The skilled artisan would recognize possession of a 24-31 subgenus in view of the written description on pages 4-6 of the specification as filed.

While formally a separate issue, it is still germane and noteworthy that this Examiner has recently stated, for the benefit of the Board of Patent Appeals and Interferences, that chimeric and humanized antibodies were enabled in the art as of early 1992. *See*, March 30, 2005 Office action in Application No. 09/467,217 at page 3 ("Therefore, chimeric and humanized antibodies are deemed enabled at the time the invention was made, which is the filing date of the earliest priority application USSN 07/835,799, filed 2/14/92"). Applicant agrees. In the current context,

where the earliest priority date claimed is over three years later, there can be no question of enablement.

Written description for the 24-31 subgenus is also expressly provided by WO 96/06666, which disclosure is incorporated by reference on page 6, lines 17-18 of the instant specification. WO 96/06666 provides in pertinent part (page 4, lines 18-20):

> More preferably, the anti-human gp39 mAb binds an epitope recognized by monoclonal antibody 24-31 . . . .

The same disclosure is found in at col. 4, lines 16-18 of US 5,747,037, of record in this application (a US equivalent of WO 96/06666).

The specification further cites to WO 92/06193 as a preferred method for production of antibodies for use in the methods of the invention. This PCT describes using the hypervariable regions (complementarity determining regions, or CDRs) of non-human origin and framework regions of human origin in the production of humanized antibodies (see, paragraph bridging pages 5-6 of the instant specification). Finally, this Examiner has previously allowed similar epitope subgenus language, with analogous support, i.e. in claim 16 of the '868 patent to Lederman. The disclosure as filed thus clearly contemplates a subgenus of antibodies capable of specifically binding the 24-31 epitope.

In view of the foregoing, it is respectfully submitted that the written description rejections have been obviated or overcome. Accordingly, withdrawal of these rejections is respectfully requested.

Rejection Under 35 U.S.C. § 112 - Indefiniteness

The examiner maintained rejection of claims 12-14 as allegedly indefinite in the recitation of "T cell mediated autoimmune responses associated with type I diabetes." The Examiner maintained that the phrase is allegedly ill-defined and ambiguous. The evidence previously submitted with the April 1, 2005 reply was not considered since it could not be located.

In response, the evidence previously submitted under Exhibit A, four abstracts including the Rabinovitch Abstract, is now being resubmitted in the form of the full-length references for completeness and the convenience of the Examiner. These references are:

Gelber C, Paborsky L, Singer S, McAteer D, Tisch R, Jolicoeur C, Buelow R, McDevitt H, Fathman CG, 1994 Jan, "Isolation of nonobese diabetic mouse T-cells that recognize novel autoantigens involved in the early events of diabetes," Diabetes 43, 33-9 ("Gelber") (**Exhibit 1**).

Doukas J, Mordes JP, 1993 Feb 1, "T lymphocytes capable of activating endothelial cells in vitro are present in rats with autoimmune diabetes," J Immunol 150, 1036-46 ("Doukas") (**Exhibit 2**).

Horio F, Fukuda M, Katoh H, Petruzzelli M, Yano N, Rittershaus C, Bonner-Weir S, Hattori M, 1994 Jan, "Reactive oxygen intermediates in autoimmune islet cell destruction of the NOD mouse induced by peritoneal exudate cells (rich in macrophages) but not T cells, Diabetologia 37, 22-31 ("Horio") (**Exhibit 3**).

Rabinovitch A, 1994 May, "Immunoregulatory and cytokine imbalances in the pathogenesis of IDDM. Therapeutic intervention by immunostimulation?," Diabetes 43, 613-21 ("Rabinovitch") (**Exhibit 4**).

Gelber, Doukas, Horio and Rabinovitch, together with the instant specification, show that the quoted phrase had clear meaning to the skilled artisan as of the earliest filing date in 1995. In fact, "T cell mediated autoimmune responses associated with type I diabetes" were known and included, e.g., insulinitis and pancreatic beta cell destruction (a/k/a islet cell destruction) (see Gelber, Doukas, and Horio), as well as the autoimmune response mediated by the T-helper-1 Th1) subset of T cells secreting pro-inflammatory cytokines, such as interleukin-2 (IL-2) and interferon gamma (IFNγ) (see Rabinovitch). The specification teaches that the methods of the invention, within which context this phrase must be viewed, "pertain to treatments of autoimmune disorders in which tissue destruction is primarily mediated through activated T cells and immune cells other than B lymphocytes." Spec. at page 3, last ¶.

This language is not indefinite since it had clear meaning to the artisan at the time. "Some latitude in the manner of expression and the aptness of terms should be permitted even though the claim language is not as precise as the examiner might desire." MPEP § 2173.02. Here, a

reasonable degree of clarity and particularity is provided, when this phrase is properly viewed in the context of the claim as a whole and in light of the specification, the teachings of the prior art, and the claim interpretation that would be given by the artisan of ordinary skill. *Id.* This language "reasonably apprise[s] those skilled in the art of both the utilization and scope of the invention" and is "as precise as the subject matter permits." *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1385 (Fed. Cir. 1986). That is all that is required. Hence, it is definite. A person skilled in the field of the invention would reasonably understand the claim when read in the context of the specification. *Miles Labs., Inc. v. Shandon*, 997 F.2d 870, 875 (Fed. Cir. 1993). Accordingly, reconsideration and withdrawal of this rejection is proper and is respectfully requested.

Rejections Under 35 U.S.C. § 103 - Obviousness

The Examiner maintained rejection of claims 1, 5-10, 12-14, 17 and 19 as allegedly obvious over the '868 patent to Lederman (i.e. 6,592,868), in view of the '037 patent to Noelle (i.e. 5,747,037). The Examiner relied on "diabetes mellitus" as mentioned in the Lederman '868 disclosure, in view of the '037 teaching of monoclonal antibody (mAb) 24-31.

The rejection is respectfully traversed.

The Examiner cited generally to the teachings of these references in finding a reasonable expectation of success for preventing T cell mediated tissue damage associated with type I diabetes. However, there is no disclosure in either cited reference to support such a reasonable expectation for any T-cell-mediated autoimmune disease. The Examiner's argument states (Office action at 5):

> In this case the teachings of both the primary and secondary references indicate success in treating diabetic patients with anti-CD40 ligand antibodies in the face of having to solve the same or nearly the same problem [and] would have led one of ordinary skill in the art at the time the invention was made to combine the references to treat the same or nearly the same diabetic patient populations with antagonistic therapeutic anti-CD40 ligand antibodies to dampen the well known inflammatory problems associated with diabetic patients in the art.

However, no specific citations to these references are provided for any teaching or suggestion which would "indicate success" in preventing T cell mediated tissue damage in diabetes type I. Lederman '868 mentions diabetes mellitus only in the context of inhibiting B cell activation (discussed below). The '037 patent does not mention diabetes at all. Neither reference suggests that an effective amount for B cell inhibition would be the same or nearly the same as a prophylactically effective amount for preventing T cell mediated tissue destruction associated with type I diabetes, as claimed.

According to the Examiner's reasoning, any anti-gp39 antibody described subsequent to 5c8 may have been obvious to try in the treatment or prevention of any immune disease. Obvious to try, of course, is not the correct legal standard. *In re O'Farrell*, 853 F.2d 894, 903 (Fed. Cir. 1988). The references must provide a reasonable expectation of success to support a *prima facie* case of obviousness. *Id.* This expectation is not provided in an unpredictable art, absent some indication of actual efficacy for the specifically claimed method. The instant specification provides exemplary results in a working example for an appropriate T cell mediated autoimmune disease model (EAE). This demonstration of efficacy cannot be used in hindsight to render the invention obvious. *In re Dow Chemical Co.*, 837 F.2d 469 (Fed. Cir. 1988). Neither the '037 nor the '868 make such a demonstration.

Thus, even assuming *arguendo* that one of ordinary skill may have been motivated to combine or modify the teachings of '868 in view of '037, the artisan would still lack a reasonable expectation of success in treating T-cell-mediated type I diabetes with mAb 24-31 from these early teachings. The references do not establish a *prima facie* case, which requires this expectation. MPEP § 2163. The expectation must be found in the prior art, not from the applicant's disclosure. *In re Vaeck*, 947 F.2d 488 (Fed. Cir. 1991).

The combined references also do not teach or suggest all of the claim limitations. The Examiner appears to acknowledge this in discussing the references at the bottom of page 4 of the Office action (" ... nor describe "T cell mediated autoimmune responses" per se ... ") (emphasis in original). Thus, neither "preventing T cell mediated tissue destruction associated with type I diabetes" as recited in claim 1, nor "preventing a T cell mediated autoimmune response associated

with type I diabetes" as recited in claim 12, is disclosed or suggested by the cited references, alone or in combination, according to the Examiner's own reading. Indeed, the specific concept of preventing T cell mediated damage in type I diabetes is missing.

The April 1, 2005 reply provided evidence in the form of a Noelle Declaration, **Exhibit D**, and other documents, **Exhibits B-C**, demonstrating that the 24-31 antibody provides superior results *in vivo* over 5c8. This evidence has not been accorded its due weight. In addition to being therapeutically safe by not causing thromboses (contrary to hu5c8), the antibodies of the presently claimed methods block the binding of CD40 to gp39 *in vivo* more effectively than 5c8. Due consideration is required of objective evidence of superior results. *Stratoflex Inc. v. Aeroquip*, 713 F.2d at 1538 ("[E]vidence arising out of the so-called 'secondary considerations' must always when present be considered en route to a determination of obviousness.").

Regarding the Lederman B cell disclosure, the Examiner maintained that "Lederman et al. is <u>not</u> limited to treating B cell immune responses only, given its teaching of inhibiting transplant rejection and autoimmune diseases such as diabetes" (Office action at 4; emphasis in original). The context of the specific Lederman '868 disclosures cited and relied upon actually demonstrates the opposite: The Lederman invention is only directed to inhibiting B cell activation, not T cell mediated autoimmune diseases. This is shown by the following Lederman '868 disclosure (col. 10, line 62 to col. 11, line 7) (emphasis added):

> This invention provides a **method of inhibiting B cell activation** in an animal which comprises administering to the animal an effective inhibiting amount of a pharmaceutical composition comprising the monoclonal antibody which specifically recognizes the activated T cell surface protein and a pharmaceutically acceptable carrier. For the purposes of this invention, an "effective inhibiting amount" of a pharmaceutical composition is any amount of the pharmaceutical composition which is effective to bind to a protein on the surface of activated T cells and thereby inhibit T cell activation of B cells. In one embodiment of this invention, the B cells are resting B cells. In another embodiment of this invention, the B cells are primed B cells.

Thus, the invention is described *strictly* in terms of inhibiting B cell activation. There is no other disclosure in Lederman or the combined references that expands the scope of this passage. Even the effective amount of monoclonal antibody to be used is described in terms of

inhibiting the activation of B cells. There is no other teaching or suggestion in Lederman or the combined references of a prophylactically effective amount for the prevention of a T cell mediated autoimmune damage in type I diabetes.

The context in which the Lederman disclosure mentions "diabetes" and "transplant" is also *exclusively* in the context of inhibiting B cell activation, not in the context of preventing a T cell mediated autoimmune disorder ('868 patent at column 11, lines 18-35) (emphasis added):

> The method of **inhibiting B cell activation** is valuable in a new and useful method for inhibiting the immune response of an animal. In one embodiment of this invention, the animal is a mammal, for example a mouse or a human. Preferably, the mammal is a human.
> In one embodiment of this invention, inhibiting the immune response of an animal is valuable as a method of inhibiting the rejection by the animal of a **transplant** organ, for example, a heart, kidney or liver.
> In another embodiment of this invention, inhibiting the immune response of an animal is valuable as a method of inhibiting the autoimmune response in an animal suffering from autoimmune disease. Examples of autoimmune diseases include, but are not limited to, rheumatoid arthritis, Myasthenia gravis, systemeic lupus erythematosus, Graves' disease, idiopathic thrombocytopenia purpura, hemolytic anemia, **diabetes mellitus** and drug-induced autoimmune diseases, e.g., drug-induced lupus.

Thus, once again, there is no mention of preventing *T cell mediated* autoimmune damage in type I diabetes. Contrary to the Examiner's assertions, the Lederman disclosure is *strictly* limited to inhibiting B cell activation. The teachings of the '037 patent, discussed at length in the prior reply, cannot cure this deficiency of Lederman '868. The mere mention of "diabetes mellitus" and "transplant" rejection in Lederman '868 simply cannot transform a strictly B cell inhibition disclosure into an adequate teaching or suggestion for use of a specific, superior, anti-gp39 antibody in a specific T cell mediated autoimmune disorder, type I diabetes.

A fair reading of Lederman '868 merely implies that diabetes mellitus and transplant rejection have B cell mediated components to which 5c8 may be applied. Indeed, it was known in the art at the time that the immune response in transplant rejection "includes humoral and cellular components," and that, in the humoral component, "B lymphocytes form antibodies to the HLA alloantigens." See, van Rood & Claas, 1990, Science 248, 1388-93 (at abstract; available via Pub

Med at www.ncbi.nlm.nih.gov). Similarly, autoimmune diabetes was known at the time to have B cell mediated and T cell mediated components. See, Voorby et al., 1990, Clin Exp Immunol 82, 542-7 (at abstract; available via Pub Med at www.ncbi.nlm.nih.gov) ("Islet-specific autoimmune reactivity (humoral and cell-mediated) is the basis for the insulitis process of type I diabetes mellitus."). Thus, the Lederman '868 patent is fairly read as limited to teaching a treatment for the B cell mediated components of disease.

The Examiner has also not shown how a B cell mediated disease component could have been reasonably expected to have the same or nearly the same "effective amount" for treatment from the teachings of the cited references. The "prophylactically effective amount" requirement recited in the claims is specifically directed to the prevention of a T cell mediated autoimmune component of type I diabetes.

Accordingly, reconsideration and withdrawal of the obviousness rejection is proper and is respectfully requested.

CONCLUSION

In view of the foregoing amendments and remarks, applicant believes the pending application is in condition for allowance. Favorable action is respectfully requested. In the event that any issue remains in connection herewith, the Examiner is respectfully invited to telephone the undersigned to discuss same.

Dated: August 15, 2005   Respectfully submitted,

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diabetes

Isolation of Nonobese Diabetic Mouse T-Cells That Recognize Novel Autoantigens Involved in the Early Events of Diabetes

COHAVA GELBER, LISA PABORSKY, STEVEN SINGER, DEBORAH McATEER, ROLAND TISCH, CHRISTINE JOLICOEUR, ROLAND BUELOW, HUGH McDEVITT, AND C. GARRISON FATHMAN

Insulin-dependent diabetes mellitus (IDDM) is thought to result from chronic, cell-mediated, autoimmune islet damage. Our aim was to identify the earliest T-cell autoantigen in IDDM, reasoning that this antigen could be causally involved in the initiation of the disease. Identification of the earliest β-cell-specific autoantigen is extremely important in allowing advances in prevention and treatment of initial events in the development of inflammatory insulitis that precedes β-cell destruction and overt diabetes. Therefore, we analyzed the proliferative responses of peripheral T-cells from young, female nonobese diabetic (NOD) mice to extracts of pancreatic β-cell lines. We were able to demonstrate that T-cells responsive to β-cell antigens exist in the peripheral lymphoid tissue of these mice in the absence of deliberate priming before the manifestation of histologically detectable insulitis. T-cell lines and clones isolated from the peripheral lymphatic tissues of young, unimmunized, female NOD mice were also shown to react with extracts of β-cells. Fractionation of the β-cell extracts showed that these T-cell clones recognized multiple β-cell-specific autoantigens but none of the previously reported putative autoantigens (glutamic acid decarboxylase [GAD]65, GAD67, Hsp65, insulin, ICA 69, carboxypeptidase-H, and peripherin). Thus, we can conclude that these responses are specific for novel β-cell autoantigens. Finally, NOD T-cell proliferative responses were also seen to an extract of human islets suggesting potential shared antigenic determinants between human and mouse β-cells. Our observation that human and murine β-cell-specific antigens are conserved offers the possibility that identification of these murine autoantigens may lead to the discovery of the human homologue. This will pave the way toward effective diagnosis and/or immunotherapy to prevent diabetes. *Diabetes* 43:33–39, 1994

uman insulin-dependent diabetes mellitus (IDDM) is caused by an autoimmune destruction of the pancreatic β-cells (1,2). Nonobese diabetic (NOD) mice spontaneously develop diabetes resembling human IDDM (3), characterized by a progressive lymphocytic infiltration of islets (insulitis) before the manifestation of overt diabetes (hyperglycemia) (4,5). Lymphocyte infiltration of islets can be detected as early as 4–6 weeks of age (6). Although antibodies directed against islet cells appear in the serum during the development of insulitis (7), NOD mouse diabetes (and presumably human IDDM) has been shown to be a T-cell-dependent disease (8–20). Susceptibility to the development of diabetes in mice (and humans) is strongly associated with the major histocompatibility complex (MHC) class II genes: mouse I-Aβ and human DQB (21–24).

This association allows the possibility that a single autoantigen might be recognized leading to infiltrative insulitis with resultant β-cell destruction and IDDM. The autoantigen(s) responsible for triggering the initial attack on the pancreatic β-cells has not yet been identified. Although the actual mediators of β-cell destruction are unknown, two previous reports have described potential targets of T-cell clones: *1*) NOD T-cell clones responded to a 65,000-M_r heat shock protein (Hsp65) and success-

fully transferred insulitis and transient hyperglycemia to prediabetic NOD mice (20), 2) a human T-cell clone derived from a newly diagnosed IDDM patient was stimulated by a 38,000-M_r protein derived from insulin secretory granules of rat insulinoma tissue (25). In addition, autoantibodies from IDDM patients and from NOD mice have identified other potential targets of the immune response (glutamic acid decarboxylase [GAD: 65,000 and 67,000 M_r], insulin, Hsp65, carboxypeptidase-H, peripherin [26–34], and ICA 69 [M. Pietropaolo, L. Castano, S. Baba, S. Martin, A. Martin, A. Powers, N. Prochazka, J. Naggert, E.H. Leiter, G.S. Eisenbarth, unpublished observations, islet cell autoantigen 69,000 M_r [ICA 69]. Molecular cloning and characterization of novel diabetes associated autoantigen.)

In an attempt to identify the events involved in the initiation of NOD disease, we studied the spontaneous T-cell proliferative response of peripheral lymphatic tissue from young, (prediabetic) NOD mice to extracts of an insulinoma cell line. T-cell proliferative responses to whole cell extracts (WCE) and subcellular fractions of the insulinoma were seen in NOD mice ≥8-days-old. NOD T-cell proliferative responses were also seen to an extract of human islets suggesting potential shared antigenic determinants between human and mouse β-cells.

T-cell clones derived from young, prediabetic NOD mice also responded to the WCE and the same subcellular fractions of the insulinoma as did peripheral lymphocytes but did not respond to purified autoantigens including GAD (65 or 67), ICA 69, carboxypeptidase-H, peripherin, insulin, or Hsp65. These data suggest that novel antigens of islet β-cells are recognized early in NOD disease and that such antigens may be shared between human and mouse β-cells. Identification of these novel autoantigens may lead to immunotherapy and/or early diagnosis of IDDM.

RESEARCH DESIGN AND METHODS

Mice. NOD mice were purchased from Taconic (Germantown, NY). Of the female mice, 80% developed spontaneous diabetes at ≤160 days. BALB/c and C57BL/6 mice were also purchased from Taconic.

Cell lines. The β-insulinoma cell line B23720 was developed from RIP-Tag2 transgenic mice containing SV-40 T antigen under the control of the rat insulin promoter (35). These mice were crossbred with NOD mice. After the eighth backcross, an insulinoma tumor was isolated from one mouse, and an in vitro cell line (B23720) was generated. These insulinoma cells express insulin, peripherin, and class I MHC but do not express GAD 65, GAD 67, or class II MHC molecules (S. Singer, H.O. McDevitt, C. Joliceour, D. Hanahan, and H. Acha-Orbea, unpublished observation).

The α-glucagonoma cell line (αTCL) was generated similarly in transgenic mice containing the SV-40 T-antigen under the control of the glucagon promoter (35), but was not crossed to NOD.

The human neuroblastoma line SY5Y was a gift from Dr. L. Jensen (UCSF, San Francisco, CA).

Human Islets. WCE was prepared by sonication of 10^9 cells in 200 μl of phosphate-buffered saline (PBS) and 3 times for 10 s on ice. Human islets were received as a gift from Dr. Ricardi (Pittsburgh, PA).

High-performance electrophoretic chromatography (HPEC) fractionation. B23720 were grown in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS) (Gibco). Cytosolic and membrane extracts from this cell line were prepared by sonication followed by a 150,000 g speed spin. B23720 cytosolic and membrane extracts were fractionated using the HPEC (ABI, HPEC, model 230A, Foster City, CA) system. A sample containing 400 μg of cell-extract in 7.5 mM tris-phosphate, pH 7.5, 0.25% sodium dodecyl sulfate (SDS), and 15% glycerol was loaded onto a 10% SDS-tris phosphate tube gel (3.5 × 10 cm) and electrophoresed using a trisphosphate buffer system. The proteins were eluted from the bottom of the gel into 7.5 mM tris HCl, pH 7.5. The collected fractions were assayed for protein concentration and then analyzed on a 12.5% SDS-polyacrylamide gel.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) fractionation of β-insulinoma proteins. Two milligrams of the insulinoma cytosolic or membrane extract was electrophoresed on a nonreducing 10% SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was cut into several strips that were sonicated in PBS to generate suspensions of fine particles that were then used in T-cell proliferation assays.

Proliferation assay. Single-cell suspensions from spleens (6–8 mice/group; pooled together) were prepared in complete RPMI-1640 medium supplemented with 5% FCS and 10 U/ml penicillin/streptomycin. Spleen cells were assayed by titrating antigen (0.3–2.0 μg/ml) into microtiter plates containing 1 × 10^6 cells in complete media, in triplicates, and harvesting as described below. For analysis of clones, cultures of 1–3 × 10^5 T-cells and 0.5 × 10^6 irradiated (2,000 R) spleen antigen presenting cells per well were cultured in triplicate with added antigen (0.3–20 μg/ml). The cultures were incubated for 72 h, pulsed with 1 μCi per well ^{3}H-methyl-thymidine (^{3}H-TdR) (Amersham, Arlington Heights, IL), and harvested 16 h later. The incorporated radioactivity was determined using a Betaplate scintillation counter (Pharmacia, LKB, Piscataway, NJ). Results are expressed as mean counts per minute of incorporated ^{3}H-TdR. Standard deviations were ≤10% of the mean.

The insulinoma extracts fractionated by HPEC were used at a protein concentration (final) of 10 μg/ml, and the samples were then diluted 1:10–1:40 to reduce toxicity caused by SDS. Similarly, when insulinoma membranes were fractionated by SDS-PAGE and blotted on nitrocellulose, the strips were sonicated, diluted 1:40–1: 1600, and tested in the T-cell assay. All presented experiments were repeated at least 3 times. The presented graphs represent results of ≥3 experiments.

Generation of β-cell-specific T-cell lines and clones. Lymphocytes from spleen or pancreatic lymph nodes taken from 30- to 40-day-old female NOD mice were cultured for 3 days with 10 μg/ml of the insulinoma WCE or membrane extracts in complete RPMI with 5% FCS. Three days later the cells were washed and cultured in complete RPMI medium containing 10% FCS and 20

U/ml recombinant-mouse IL-2 (Genzyme, Cambridge, MA).

The T-cell lines were established by repeated cycles of stimulation as described above and cloned by standard limiting dilution procedures. Growing clones were expanded and tested for antigen recognition in proliferation assays.

Expression and purification of recombinant proteins.
The cDNA encoding human Hsp65 was cloned by polymerase chain reaction (PCR) using polyA$^+$RNA isolated from a human Epstein Barr Virus–transformed β-cell line (Priess) (36). Postincubation was at 42°C for 2 h. Following reverse transcription, the Hsp65 encoding DNA fragment was amplified by PCR using the primers 5'CGG GGATCCGCCAAAGATGTAAAATTTGGTGCAGATGCC and 5'GTCCTCGAGTTAGAACATGCCACCTCCCATAC CACCTCC (30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C). The cDNAs encoding for human carboxypeptidase-H, ICA69 (gifts from G. Eisenbarth), and Hsp70 (37, ATCC/clone pH 2.3) were cloned into expression vector pTrc99A (His6) that was constructed by insertion of a synthetic DNA fragment encoding six histidine residues ([CAC]6) into the polylinker of the pTrc99A expression vector (38). The recombinant proteins were tagged with six histidine residues at the NH$_2$-terminus. Plasmid constructs were transformed into E. coli-TG1 (supE hsdΔslac-proAB) F'[traD36proAB + lacIqlacZΔM15] and protein expression was induced by addition of IPTG to the culture medium. Bacteria were lysed in 100 mM Tris pH 8.0, 6M GuHCl and insoluble material was removed by centrifugation at 40,000 g for 30 min. Recombinant proteins were purified using Ni-NTA-agarose (Qiagen, Chatsworth CA) in the presence of 6 M GuHCl and dialyzed against PBS. Protein concentration was determined using BCA (protein assay reagent) assay (Pierce, Rockford, IL).

Full-length murine cDNAs encoding the two isotypes of GAD (GAD65, GAD67), peripherin, and carboxypeptidase-H were engineered to contain six histidine residues found at the COOH-terminus, thereby allowing affinity purification of each antigen using a Ni^{++}-conjugated resin.

A baculovirus expression system has been used to express the cDNAs as recombinant proteins. Briefly, the histidine-tagged cDNAs were subcloned into the PVL 1393 transfer vector and transfected into Sf9 cells with baculovirus. Recombinant viruses were selected and assessed for protein expression. Selected viral recombinants were then used for large-scale expression of each protein. Pancreatic hormones were purchased from Sigma (Sigma, St. Louis, MO). The rat C-peptide sequence was EVENPQVPQIGGGPEAENIQTIAIEVARQ, and the mouse C-peptide sequence was EVEDPQVEQ IGGSPGDIQTIAIEVARQ (28–31).

RESULTS

Unprimed peripheral lymphocytes from young, female NOD mice of different ages (Fig. 1A) were isolated from spleen and stimulated in vitro by whole cell, cytosolic, or membrane extracts from the B23720 (insulinoma) cell
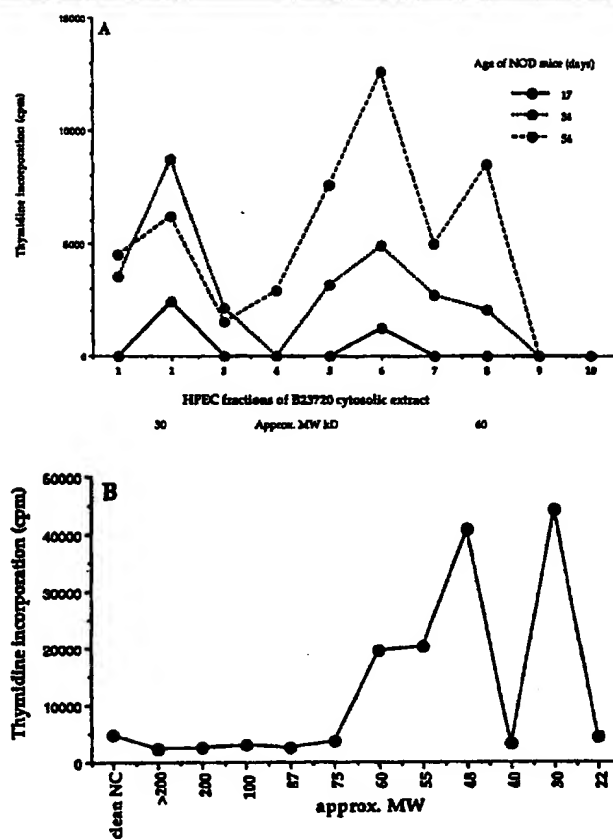

FIG. 1. T-cell response to fractionated β-cell antigens in young NOD mice. A: Insulinoma cytosolic extracts fractionated by HPEC (ABI, HPEC, model 230A) were used in a proliferation assay testing the reactivity of splenocytes obtained from 17- to 54-day-old female NOD mice. B: Insulinoma membrane extracts, separated by SDS-PAGE and transferred to nitrocellulose, were cut and sonicated to yield small particles that were used in a T-cell proliferation assay to test the response of spleen cells from 24-day-old female NOD mice.

line. T-cell proliferation to the WCE of the insulinoma was seen using cells from NOD mice at each age assayed beginning at ≥8 days old (data not shown). To separate the components of the insulinoma WCE recognized by unprimed NOD T-cells, protein fractionation of the WCE was performed based on SDS-PAGE and elution of the fractions into liquid phase by HPEC. Proliferative responses of splenocytes from 17- to 54-day-old female NOD mice were assayed using cytosolic extracts of the insulinoma fractionated by HPEC. Data from 1 of 10 similar assays are presented in Fig. 1A. Three major peaks that elicited T-cell proliferation were identified with approximate molecular weights of 30,000–40,000, 50,000, and 55,000–65,000 M_r in the cytosolic fraction of the insulinoma antigen preparation (Fig. 1A). The magnitude of the T-cell proliferative response to these fractions increased with age (Fig. 1A), but proliferative responses could be seen in female NOD mice as early as 8 days old (Fig. 2B).

When insulinoma membrane extracts were fractionated by conventional SDS-PAGE followed by blotting of the proteins on nitrocellulose and the nitrocellulose particles carrying the proteins were assayed by T-cell pro-

FIG. 3. β-cell-specific antigens are conserved across species. WCE of human islets were fractionated by HPEC and tested in a proliferation assay with unprimed NOD spleen cells from 40-day-old female mice.
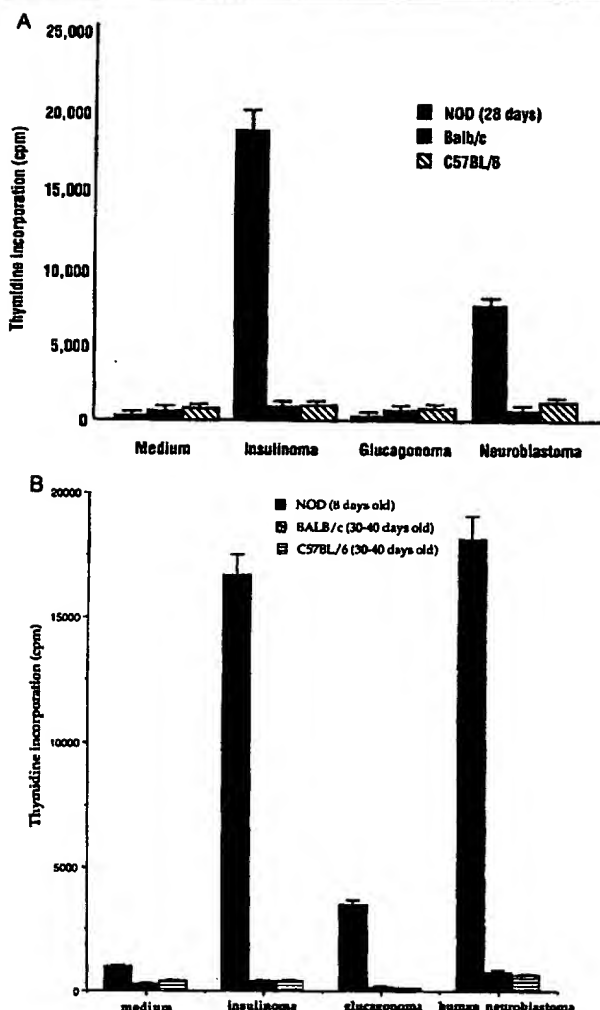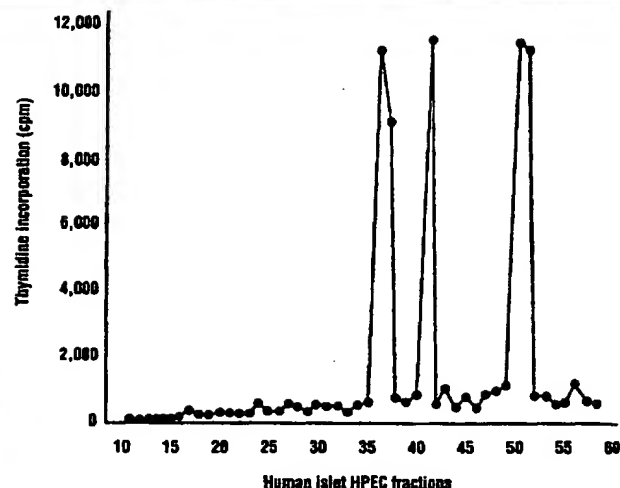
FIG. 2. The T-cell proliferative response to murine insulinoma and human neuroblastoma antigens can be demonstrated in NOD mice and is absent in nondiabetic mouse strains and early NOD T-cell responses to β-cell antigen. A: Splenocytes from a 28-day-old female NOD and a 30- to 40-day-old BALB/c or C57BL/6 mice were tested against HPEC fractionated antigen (10 mg/ml) from the insulinoma (B23720), the glucagonoma (αTCL), and human neuroblastoma (SY5Y). HPEC fractions within the size range of 30,000–60,000 M, were pooled and used in the assay. B: Splenocytes from 8-day-old female NOD mice and 30- to 40-day-old BALB/c or C57BL/6 mice were tested as described above.

liferation using spleen cells from 24-day-old female NOD mice, a similar pattern of T-cell activation was obtained (Fig. 1B). Thus, the autoantigens can be detected in the membrane and in the cytosol of the B23720 insulinoma cell line.

To demonstrate specificity, the proliferative responses of splenocytes from a 28-day-old NOD, from a 30- to 40-day-old BALB/c, and from C57BL/6 female mice (used as control samples) were compared using HPEC fractionated cytosol preparations from 1) the NOD insulinoma (B23720), 2) a murine α-glucagonoma (αTCL), and 3) a human neuroblastoma (SY5Y). The proliferative response seen in NOD mice to cytosolic proteins of the insulinoma was specific (Fig. 2): 1) T-cells obtained from

the same NOD mice failed to respond to glucagonoma proteins, and 2) splenocytes from other nondiabetic mouse strains including BALB/c and C57BL/6 did not respond to the insulinoma extract (Fig. 2A). Furthermore, when splenocytes from 8-day-old female NOD mice were tested, a spontaneous proliferative response to β-cell but not to α-cell antigens was detected (Fig. 2B). Control mice T-cell proliferative responses to alloantigens and mitogens were found to be similar in cells from all mice tested (data not shown). These data suggest that the T-cell proliferative response to the insulinoma is specific and seen only in NOD mice. Moreover, these data demonstrate that T-cells responsive to β-cell antigens exist in the peripheral lymphoid tissue of very young, female NOD mice in the absence of deliberate priming and before manifestation of histologically detectable insulitis.

Because the cytosolic preparation from the human neuroblastoma (SY5Y) also triggered NOD T-cell proliferation (Fig. 2), these results suggest that these neuroendocrine-derived cells may share antigenic determinants with the insulinoma and such determinants may be conserved across species. To further address the potential for β-cell-specific, antigenic determinants shared across species, human islets were obtained from which a WCE was prepared, fractionated by HPEC, and assayed in a NOD T-cell proliferative assay. Human islet cell–derived proteins, with approximate molecular weights of 30,000–40,000, 50,000, and 55,000–65,000 M, were shown to induce proliferation of T-cells from spleens of 40-day-old, unprimed, female NOD mice (Fig. 3). This result indicates that β-cell-specific antigens may be conserved between humans and mice that can be recognized by unprimed T-cells from NOD mice.

To determine whether proteins contained in the three peaks that correspond to T-cell-stimulating activity (Fig. 1) were the result of a degradation process (1), represented isoforms of the same molecule (2), or represented
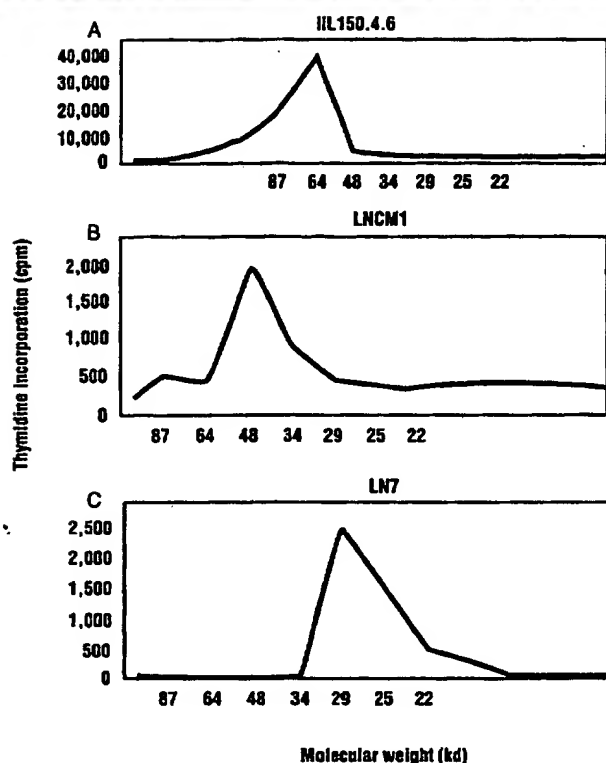
FIG. 4. Existence of several discrete β-cell-specific autoantigens detected by the peripheral T-cell response in the NOD mouse. Proliferative responses of three insulinoma-specific T-cell clones tested with the SDS-PAGE fractionated β-cell extract. (IIL150.4.6 [A], CD4$^+$ T-cell cloned from islets; LNCM1 [B] and LN7 [C], CD4$^+$, I-A^{NOD} restricted T-cells were cloned from lymph-nodes of 30-day-old female NOD mice).

FIG. 5. Antigen specificity of T-cell line and clones from young, female NOD mice (representative graph). The antigen specificity of insulinoma-specific T-cell line was tested in a proliferation assay using whole-cell extracts of islets, insulinoma, and pancreatic hormones (upper and middle panel). T-cell hybridomas generated from the above T-cell line were assayed with recombinant proteins in cytokine (IL-2) release assays. (The assays were performed with a protein concentration of 10µg/ml).

different antigens (3), a panel of insulinoma-specific T-cell clones was generated and tested against the SDS-PAGE fractionated insulinoma extract. Insulinoma-specific T-cell lines established from lymph node cells of 30-day-old female NOD mice selected on insulinoma WCE exhibited a profile of T-cell reactivity similar to unprimed NOD lymphocytes, i.e., responses corresponding to the three peaks of activity within the molecular weight range of 30,000–60,000 M_r (data not shown). The clones were generated following two cycles of antigenic restimulation with insulinoma WCE. The T-cell lines were cloned by limiting dilution. Different T-cell clones (CD4$^+$, I-A^{NOD} restricted) proliferated in response to either the 30,000-M_r fraction or to the 50,000-M_r fraction of insulinoma antigen (Fig. 4C and B, respectively). These T-cell clones were shown to accelerate destructive insulitis upon adoptive transfer into 14-day-old NOD mice (data not shown) suggesting their relevance to the disease process. A third β-cell-specific, CD4$^+$- and I-A^{NOD}-restricted T-cell clone was isolated from islets of 30-day-old NOD mice previously infused with diabetic T-cells isolated from spleens. This islet-infiltrating T-cell clone was shown to be stimulated by the 65,000-M_r fraction of insulinoma antigen (Fig. 4A). These data suggest that the insulinoma proteins contained within the peaks of T-cell activity, against WCE, probably consist of

discrete proteins. Furthermore, these data suggest that T-cells from young, prediabetic, female NOD mice react with more than one insulinoma antigen.

To determine whether T-cells from unprimed, female NOD mice were responding to previously identified candidate autoantigens, a T-cell line generated from pooled lymphocytes from eight 30-day-old, female NOD mice and the three T-cell clones (Fig. 4) were challenged with a panel of antigens including cell extracts (islets, insulinoma, and glucagonoma), pancreatic hormones (insulin [28–31], glucagon, and somatostatin), proinsulin peptides (28–31), and recombinant proteins (Hsp65 [20, 32], carboxypeptidase-H [33], peripherin [34], GAD65, GAD67 [26,27], and ICA69). All tested T-cell clones and the line from 30-day-old female NOD mice were vigorously stimulated by β-cell extracts (islet and insulinoma) but not by α-cell extracts, pancreatic hormones, or any of the above mentioned recombinant proteins (Fig. 5). Spontaneous T-cell proliferative responses of NOD lymphocytes to the recombinant proteins Hsp65, peripherin, GAD65, GAD67, and carboxypeptidase-H have been demonstrated although not earlier than 4 weeks of age (R. Tisch, unpublished observations).

DISCUSSION

Data presented in this study suggest that young, female NOD mice ≥8 days old have peripheral autoreactive T-cells that recognize novel islet cell antigens in the absence of antigen priming and preceding the onset of histopathologically demonstrable insulitis. Furthermore, these autoreactive, NOD T-cells recognize human islet cell extracts suggesting conservation of antigenic determinants between human and murine islet β-cells. The

autoreactive, T-cell proliferative responses observed can be seen using splenocytes from very young (8-day-old), female NOD mice (Fig. 2B). The inability of peripheral lymphocytes from young NOD mice to recognize conventional autoantigens including GAD65, GAD67, ICA69, Hsp65, carboxypeptidase H, and peripherin, and the inability of such T-cells to respond to insulin, suggests that recognition of novel β-cell autoantigens might precede β-cell damage with resultant release and recognition of these putative autoantigens. These data are consistent with the unpublished observation suggesting that peripheral lymphocytes from female NOD mice begin to recognize these conventional autoantigens between 4 and 5 weeks of age (R. Tisch, unpublished observation).

The NOD autoreactive T-cell response to SDS-PAGE or HPEC fractionated β-cell, cytosolic, or membrane extracts suggested that multiple β-cell-specific autoantigens are recognized early in NOD disease (Fig. 1). Furthermore, when isolated T-cell clones from young, female NOD mice were assayed with these antigen fractions, each responded to one of the different fractions, which suggests that the different peaks triggering T-cell reactivity were different proteins. The results we have described support the possibility that there are multiple and distinct species of β-cell-specific antigens recognized in the early inflammatory events of NOD disease and suggest that autoreactive T-cells can be identified in very young, prediabetic, female NOD mice that recognize these multiple β-cell antigens.

Knowledge of the autoantigen(s) recognized by T-cells before overt diabetes would be important for certain forms of effective immunotherapy. More importantly, these data suggest that the autoantigens first identified leading to inflammatory insulitis may not be those that have previously been characterized as autoantigens by assaying antibodies and/or T-cells from overtly diabetic humans and/or mice. Our results support the possibility that early recognition of novel β-cell-specific autoantigens leads to inflammatory insulitis with resultant β-cell destruction and the release of β-cell products whose recognition then results in the production of the more conventional autoantibodies and T-cell proliferative responses to antigens such as GAD65, GAD67, Hsp65, etc. If this hypothesis is correct, it would suggest that identification of these early β-cell-specific autoantigens is extremely important in allowing advances in prevention and treatment of early events in the development of inflammatory insulitis that precedes β-cell destruction and overt diabetes. Our observation that β-cell-specific antigens are conserved between humans and mice offers the possibility that identification of these murine autoantigens may lead to the discovery of the human homologue and pave the way toward effective diagnosis and/or immunotherapy to prevent diabetes.

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T Lymphocytes Capable of Activating Endothelial Cells in Vitro Are Present in Rats with Autoimmune Diabetes[1]

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ABSTRACT. Endothelial activation as evidenced by increased expression of leukocyte adhesion molecules occurs during immune-mediated inflammatory processes. One such process is insulitis, the pancreatic islet inflammation that leads to autoimmune insulin-dependent diabetes mellitus (IDDM). To determine if the induction of IDDM correlates with the presence of T lymphocytes capable of activating endothelial cells (EC), we studied the diabetes resistant BB (DR) rat. These animals become diabetic after in vivo depletion of T cells expressing the RT6 alloantigen. Various populations of purified DR T lymphocytes were cocultured with MHC compatible rat EC. We observed: 1) RT6$^-$ T cells from diabetic animals induced maximal endothelial MHC Ag expression. 2) The ability of RT6$^-$ T cells to activate EC increased with the duration of in vivo RT6 depletion. It was acquired before the onset of insulitis but subsided after the onset of diabetes. 3) In contrast, neither unsorted total T cells nor in vitro-purified RT6$^-$ T cells activated EC. 4) Older DR rats depleted of RT6$^+$ T cells did not become diabetic and their RT6$^-$ T cells did not activate EC. 5) T cell IFN-γ production correlated with the intensity of EC activation. 6) direct T cell-EC contact was required for maximal IFN-γ production and EC activation. We conclude that RT6$^-$ T cells capable of activating EC are generated during the induction of IDDM in DR rats. We hypothesize that such T cell activity may lead to endothelial activation in vivo and contribute to immune-mediated insulitis, β-cell destruction, and IDDM. *Journal of Immunology*, 1993, 150: 1036.

I t is well known that endothelial activation accompanies immune-mediated inflammatory reactions (1, 2). This activation is characterized by increased expression of leukocyte adhesion molecules including class I MHC, class II MHC, and ICAM-1[3] (CD54). Endothelial

activation appears to result from cytokines released by activated lymphocytes and macrophages. Hyperexpression of leukocyte adhesion molecules probably contributes to inflammation by enhancing leukocyte-EC interaction. Although the broad outlines of this process are defined, many important details remain to be clarified. In the case of autoimmune inflammation in particular, the early events that initiate endothelial activation are not known.

One autoimmune disorder associated with EC activation is IDDM. Immune-mediated inflammation of pancreatic islets, termed insulitis, is the pathologic substrate that precedes and accompanies the destruction of insulin producing β-cells in IDDM. Several lines of evidence suggest that pancreatic EC become activated during insulitis. In human IDDM, morphologic studies have shown enlarged and dilated capillaries, and immunohistochemical analyses have revealed hyperexpression of EC class I and class II MHC molecules (3, 4). In the spontaneously diabetic BB

rat model of IDDM, increased endothelial class II expression occurs before T cell infiltration of the islets (5). Altered EC morphology and hyperexpression of leukocyte adhesion molecules also occur in nonobese diabetic mice (6–8). However, in none of these cases are the events that initiate EC activation known.

To learn more about these complex in vivo processes, we studied T cell-EC interaction in vitro. T cells have been implicated in the development of both human IDDM and its principal murine models (9). T cells also represent a source of EC activating cytokines, and circulating T cells are potential targets of EC adhesion molecules.

To conduct these studies we used T cell-EC cocultures. Such cocultures are very useful in modeling in vivo events. For example, contact between human T cells and allogeneic EC in coculture induces T cell IFN-γ production and increased endothelial class I MHC, class II MHC, and ICAM-1 expression (10). EC also provide costimulatory activity to T cells, augmenting T cell IL-2 production and proliferation (11). Both membrane-bound ligands and soluble factors have been implicated as sources of endothelial costimulatory activity (12–14).

The T cells we studied were obtained from BB rats from the DR subline. Most of these animals become diabetic after in vivo depletion of the T cell subset expressing the RT6 alloantigen (15). Transfusions of DR splenocytes leading to engraftment of RT6$^+$ T cells prevent diabetes in diabetes prone BB rats (16). RT6 is expressed on ~50% of CD4$^+$ and ~70% of CD8$^+$ rat T cells (17), and it has been theorized that RT6$^+$ T lymphocytes act as suppressor cells and RT6$^-$ T lymphocytes as effector cells in IDDM. In our studies, we observed that in vivo depletion of RT6$^+$ DR rat T cells confers on the remaining RT6$^-$ population the ability to activate EC in vitro. These events preceded the appearance of insulitis, and suggest that similar occurrences could be involved in the initiation of insulitis and IDDM.

Materials and Methods

Animals

DR rats were purchased from National Institutes of Health-sponsored colonies at the University of Massachusetts Medical Center. The cumulative frequency of spontaneous diabetes among the DR animals in this colony is <1% (18). DR rat T cells express the RT6.1 alloantigenic form of RT6. Histocompatible (RT1^u) Wistar Furth rats were purchased from Charles River Laboratories (Wilmington, MA).

Except where stated, all animals were born and maintained under VAF conditions. This involved housing the animals in sterile hoods and autoclaving all cages, bedding, food, and water. After receipt, all animals were monitored serologically for maintenance of VAF status.

This was defined as the absence of circulating antibodies against Sendai virus, pneumonia virus of mice, KRV, Toolan's H-1 virus, Theiler's murine encephalomyelitis virus, reovirus type 3, lymphocytic choriomeningitis virus, mouse adenovirus, and *Mycoplasma pulmonis*. RT6 depletion of DR rats housed under VAF conditions does not lead to the induction of IDDM unless an immune system activator such as poly I:C is administered concurrently (19). Intentional infection of VAF DR rats with KRV, but not Sendai virus, also renders them subsequently susceptible to diabetes induction by RT6 depletion (20).

A subgroup of DR animals was housed under conventional conditions and became antibody-positive for KRV only. The great majority of these conventionally housed DR rats become diabetic after depletion of RT6$^+$ T cells.

Antibodies

OX-6 (anti-RT1.B class II MHC), OX-18 (anti-RT1.A class I MHC), OX-33 (directed against a form of CD45 expressed on B cells), OX-41 and OX-42 (directed against macrophages, granulocytes, and dendritic cells), and OX-54 (anti-CD2) were gifts of Alan Williams (Oxford University, Oxford, UK) and Arthur Like (University of Massachusetts). R7.3 (anti-α/β-TCR) was purchased from Bioproducts For Science (Indianapolis, IN). 1A29 (anti-rat ICAM-1) was purchased from Seikagaku Corp. (Rockville, MD). All of the above are mouse IgG antibodies. DS4.23 (anti-RT6.1) and GY1/12 (anti-RT6.2) were maintained in culture in our laboratory using RPMI containing 10% FCS, and the culture supernatants used for animal treatments. Both are rat IgG antibodies. The GY1/12 mAb was originally the gift of Dr. Geoffrey Butcher (Cambridge University).

Induction of diabetes in DR rats

DR rats 20 to 30 days of age were depleted of RT6.1$^+$ cells by daily injections of DS4.23 anti-RT6.1 mAb (2-ml tissue culture supernatant five times weekly). Certain VAF DR rats were also concurrently treated with poly I:C (Sigma Chemical Co., St. Louis, MO; 5 µg/g body weight, 3 times/wk). Diabetes was defined as the presence of glycosuria and a serum glucose concentration >200 mg/dl. Diabetic animals were treated with a single daily injection of PZI insulin to prevent ketoacidosis until used. Flow cytometry confirmed that these procedures reduced the percentage of RT6$^+$ lymph node T cells from ~60% to ≤4% of total T cells in all groups of rats. In conformity with previous observations, conventionally housed, RT6-depleted animals developed insulitis by day 10 and diabetes by day 21 (21), whereas neither insulitis nor diabetes occurred in VAF DR rats unless poly I:C was concurrently administered (19).

Histologic procedures

Pancreata from selected diabetic and nondiabetic rats were fixed in Bouin's solution and embedded in paraffin. Tissue sections stained with hematoxylin eosin were then examined for the presence of insulitis.

Endothelial cell cultures

Rat aortic endothelial cells were isolated from Wistar Furth rats. Briefly, the thoracic aorta was isolated and cleared of adventitia and the intercostal vessels clamped. The aorta was ligated below the aortic arch using a blunt-tipped 21-gauge catheter and then removed to a petri dish. After flushing with warm PBS, the distal end was clamped and the lumen filled with collagenase solution (0.5% type I collagenase, Worthington Biochemical Corp., Freehold, NJ, in Ca^{2+}, Mg^{2+}-containing PBS, 37°C). After a 30-min incubation at 37°C, the collagenase solution was recovered and the lumen flushed with PBS. The combined solutions were centrifuged at 300 × g for 5 min and the pellet then resuspended in culture medium. The medium consisted of DMEM/Ham's F12 mixture (GIBCO, Grand Island, NY) with 10% FCS, (HyClone Labs., Logan, UT), 10% Nu-Serum IV (Collaborative Research, Bedford, MA), 50 μM hypoxanthine, 50 μM 2-ME, 50 μg/ml endothelial cell growth supplement (Collaborative Research), 100 μg/ml heparin, 2 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. The cells were plated onto tissue culture plastic coated with human fibronectin (New York Blood Labs., New York, NY). Contaminating adherent cell types were removed by complement-mediated lysis using T11D7e2 anti-rat Thy-1.1 antibody (American Type Culture Collection, Rockville MD), which recognizes most contaminating cell types but not EC. Contaminating cells not lysed by T11D7e2 plus complement were physically detached using micropipette tips. Cultures were verified to be pure EC by their expression of acetylated low-density lipoprotein receptors (22). Cultures were serially subcultured to establish cell lines. Individual lines were used at passages 4 to 20, with no functional differences seen between higher or lower passages, or between different lines of EC.

T cell isolation

Cervical and mesenteric lymph nodes pooled from 4 to 18 animals were extruded through wire mesh screens to release cells. Monocytes were removed by adherence to fibronectin-coated plastic (10), and B cells were removed by adherence to 100 × 15 mm bacteriologic grade dishes coated for 12 h with anti-rat-Ig (Organon Tecknica Corp., West Chester, PA). The coating solution consisted of 100 μg antibody diluted in 10 ml 0.05 M Tris, pH 9.5. T cell viability was ≥96% as assessed by trypan blue exclusion,

and purity ≥97% as assessed by FMF analysis for CD2 and α/β-TCR expression. No monocytes (OX-41 and OX-42) or B cells (OX-33 or anti-rat IgG) were detectable. T cells isolated from anti-RT6.1-treated animals were ≥96% RT6⁻ and >80% CD4⁺.

In vitro panning (10) was also used to isolate RT6⁻ T cells (by negative selection) or RT6⁺ T cells (by positive selection) from untreated animals. Purity and viability were comparable to those achieved by in vivo depletion.

Leukocyte/EC cocultures

Purified T cells were resuspended in EC culture medium and added to confluent EC monolayers at a T cell:EC ratio of ~20:1 (1 × 10⁷ T cells/well of a six-well plate). EC treated with 100 to 300 U/ml purified glycosylated rat rIFN-γ (GIBCO) were used for positive controls for antigen expression, and EC cultured in medium alone were used as negative controls. In some experiments, Con A (3 μg/ml) was also added to cocultures at their start. After 4 days, culture supernatants were harvested for cytokine analysis and T cells removed by repeated rinses. After morphologic examination under an inverted microscope, EC monolayers were suspended using trypsin/EDTA. EC Ag expression was quantified by indirect immunofluorescence and FMF analysis using phycoerythrin-labeled anti-mouse IgG (Vector Laboratories, Burlingame, CA) as a secondary antibody. Residual T cells were excluded from analysis by selective size gating. For each Ag, 10,000 cells were examined. FMF histograms are presented as relative fluorescence intensity (X axis, determined on a three-cycle logarithmic scale and converted to a linear scale of 300 channel numbers) vs frequency (Y axis, 150 U linear scale). FMF data are also presented as the percentage of EC expressing Ag and their mean fluorescence intensity (linear scale of 300 channel numbers) corrected for non-specific binding of a purified mouse IgG (Organon Tecknica, West Chester, PA). Each experiment was repeated a minimum of three times with qualitatively similar results.

IFN-γ determination

Culture supernatants were centrifuged to remove cells and stored at −70°C. IFN-γ concentrations were measured using a rat IFN-γ ELISA (GIBCO). The limit of detectability by this method is 100 pg/ml (equivalent to 0.4 TNO Laboratory Standard Units/ml). All samples were measured in duplicate and averaged. Where values for multiple samples are averaged, values are presented as means ± SEM.

Coculture of T cells and EC in two-compartment system

In some experiments, T cells were placed inside 0.45μm microporous membrane inserts (Costar Corp., Cambridge MA) and suspended above EC monolayers. In this way T
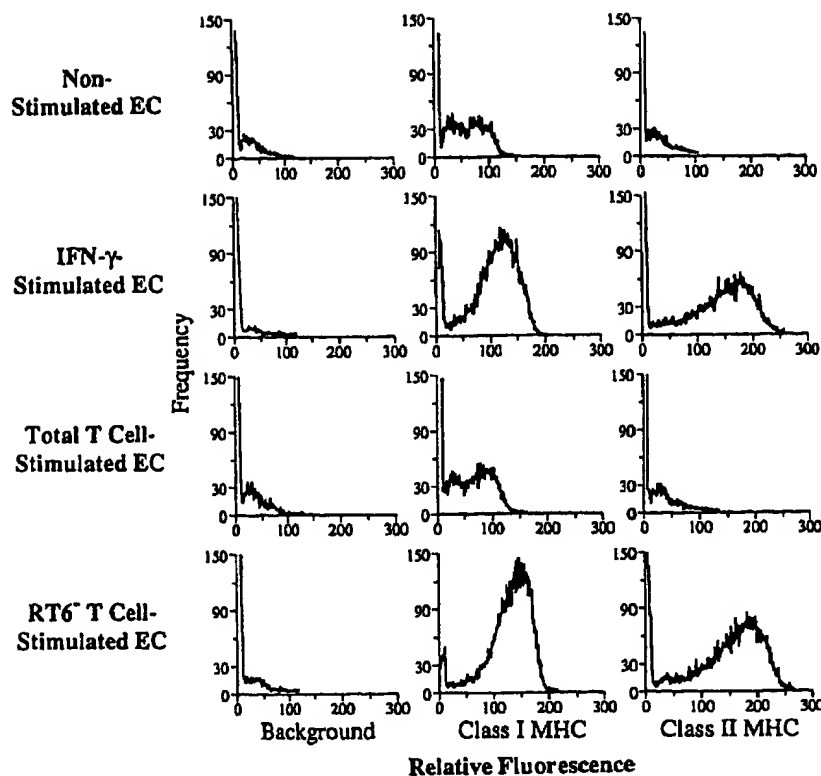
FIGURE 1. Endothelial cell MHC Ag expression induced by T cells from untreated or RT6-depleted DR rats. From *top* to *bottom*, rat EC were cultured with 1) medium alone (*Non-stimulated EC*), 2) with 200 U/ml rat rIFN-γ (*IFN-γ-stimulated EC*), 3) with total LN T cells from untreated DR rats (*Total T cell-stimulated EC*), or 4) with RT6⁻ T cells from conventionally housed DR rats treated with anti-RT6 mAb for 21 days (*RT6⁻ T cell-stimulated EC*). The RT6⁻ T cells were obtained from 10 animals that were diabetic for 1 to 4 days before use. T cells were cocultured with EC at a T cell-EC ratio of ~20:1. After 96 h endothelial MHC Ag expression was determined as described in *Materials and Methods*. Data are presented as histograms of relative fluorescence intensity vs frequency. The data shown are from a single representative experiment. The experiment was performed seven times with qualitatively similar results. A similar related experiment is presented in Table I, experiment 3. To exclude the possibility that Wistar Furth EC activation by RT6⁻ T cells was the consequence of an allogeneic (albeit MHC identical) interaction, two additional experiments were conducted using DR EC. The results (not shown) were qualitatively similar.

cells could be cocultured in the same well and medium as the EC but without direct contact with the EC monolayers. IFN-γ production and EC Ag expression were determined as described above.

Results

RT6⁻ T cells from RT6-depleted diabetic DR rats activate EC in vitro

Cultured rat aortic EC expressed low basal levels of class I and no class II MHC Ag (Fig. 1, *top row*). As expected, treatment with rat rIFNγ-induced uniform class I and class II expression on nearly all EC within a culture (Fig. 1, *row 2*). Coculture of EC with total LN T cells from untreated DR rats induced slight endothelial class I hyperexpression but no de novo class II expression (Fig. 1, *row 3*). In contrast, RT6⁻ T cells isolated from RT6-depleted

diabetic DR rats induced maximal endothelial class I and II MHC expression (Fig. 1, *bottom row*). The data shown in Figure 1 were taken from a single representative experiment that was performed a total of seven times with qualitatively similar results.

Additional evidence of endothelial activation (23) was also observed in monolayers that expressed maximal levels of class I and class II MHC Ag. This included a characteristic morphologic change from a cobblestone appearance to a swirling pattern of elongated cells.

To control for the effect of antibody administration, conventionally housed DR rats <30 days of age were treated with GY1/12 anti-RT6.2 mAb. Because BB rats express the RT6.1 allotype, these rats were not T cell depleted and did not develop diabetes. In coculture with EC, T cells from these animals were functionally similar to total T cells isolated from untreated donors. Neither of these groups induced shape changes in the EC monolayers

Table I

IFN-γ production and endothelial MHC expression (% positive cells and mean fluorescence intensity) after coculture with RT6⁻ T cells from diabetic and nondiabetic DR rats[a]

Expt.	Endothelial Cell Coculture Conditions	IFN-γ (ng/ml)	Class I MHC		Class II MHC	
			% positive[b]	Mean FI[c]	% positive[b]	Mean FI[c]
1	EC alone	<0.1	7	43	0	
	IFN-γ	ND	91	118	56	142
	Nondepleted T cells	<0.1	9	42	0	
	Nondepleted T cells + Con A	9.2	97	138	52	131
	2-day RT6-depleted T cells	<0.1	18	50	0	
	2-day-depleted T cells + Con A	7.2	92	120	35	123
2	EC alone	<0.1	12	68	0	
	IFN-γ	ND	98	142	87	171
	4-day RT6-depleted T cells	0.6	94	138	24	138
	4-day-depleted T cells + Con A	3.8	90	140	50	133
3[d]	EC alone	<0.1	30	59	0	
	IFN-γ	ND	99	159	70	161
	21-day RT6-depleted T cells	17.0	93	140	98	208
	21-day-depleted T cells + Con A	17.4	97	143	95	198
4[e]	EC alone	<0.1	4	34	0	
	IFN-γ	ND	95	116	74	150
	Nondepleted T cells	<0.1	45	66	0	
	Nondepleted T cells + Con A	11.3	84	101	62	131
	30-day RT6-depleted T cells	<0.1	42	66	0	
	30-day-depleted T cells + Con A	15.8	97	126	81	153

[a] Data from four representative coculture experiments, each of which used a single EC preparation. Each of the four experiments in this table have been repeated three to seven times with qualitatively similar results. Rat EC were cultured with medium alone (EC alone), with rat rIFN-γ (200 U/ml), or with purified DR rat T cells (T cell:EC ratio ~20:1) with or without Con A (3 µg/ml). T cells were isolated from conventionally housed DR rats that were either untreated (nondepleted T cells) or depleted of RT6⁺ T cells for various lengths of time. After 96 h of coculture, IFN-γ concentration in coculture supernatants and endothelial MHC Ag expression were determined as described in *Materials and Methods*.

[b] % = Percentage of EC expressing Ag.

[c] Mean fluorescence intensity per cell (linear scale of 300 channel numbers).

[d] Animals were used 1 to 3 days after development of hyperglycemia.

[e] Animals were used 9 to 10 days after development of hyperglycemia.

nor significant endothelial MHC Ag expression (data not shown).

To exclude the possibility that Wistar Furth EC activation by RT6⁻ T cells was the consequence of an allogeneic (albeit MHC identical) interaction, we conducted additional control experiments similar to those presented in Figure 1 using EC from DR rats. We observed that total DR T cells failed to activate DR EC, whereas RT6-depleted T cells from DR rats did induce endothelial class I and class II MHC expression to a degree similar to that observed when Wistar Furth EC were used (data not shown).

Ability of RT6⁻ T cells to activate EC varies with duration of anti-RT6 treatment

Conventionally housed DR rats <30 days of age were depleted of RT6⁺ T cells for various lengths of time before T cell isolation (Table I). In all experiments, RT6⁺ T lymphocytes were reduced to <4% of total LN cells in depleted animals. No differences in T cell viability or purity were observed after 2, 4, 21, or 30 days of depletion, but the ability of RT6⁻ T cells to activate EC varied with the duration of anti-RT6 treatment (Table I). RT6⁻ T cells from DR rats treated with anti-RT6 mAb for only 2 days were

functionally similar to T cells from untreated animals with respect to EC activation and IFN-γ production (Table I, experiment 1). Both T cell populations induced some endothelial class I MHC hyperexpression, but no de novo class II expression. As expected, given the absence of EC class II MHC expression, IFN-γ was not detectable in these cocultures. To demonstrate that both T cell populations were fully responsive to polyclonal stimulation, Con A was added directly to the cocultures and stimulated both T cell IFN-γ production and subsequent endothelial MHC Ag expression (Table I, experiment 1). Con A had no effect on endothelial Ag expression in the absence of T cells (data not shown).

In contrast, 4 days of RT6 depletion yielded RT6⁻ T cell populations that, relative to IFN-γ-treated EC, induced maximal endothelial class I and submaximal class II MHC expression (Table I, experiment 2). Addition of Con A to the cocultures further stimulated IFN-γ production and endothelial class II expression. Depletion of RT6⁺ T cells was next continued for 21 days, at which time all rats had been diabetic for 1 to 4 days. These diabetic DR rats yielded RT6⁻ T cells that induced maximal class I and maximal class II endothelial MHC Ag expression (Table I, experiment 3, and also Fig. 1, *row 4*). The addition of Con

Table II
Endothelial MHC expression (% positive cells and mean fluorescence intensity) after coculture with T cells from control, poly I:C-treated, and RT6-depleted VAF DR rats[a]

Expt.	Endothelial Cell Coculture Conditions	Class I MHC		Class II MHC	
		%[b]	Mean FI	%	Mean FI
1	EC alone	15	48	0	
	IFN-γ	96	146	37	133
	Nondepleted T cells	26	61	0	
	4-day RT6-depleted T cells	36	66	0	
	Nondepleted T cells from rats treated with poly I:C for 4 days	15	54	0	
	4-day RT6-depleted T cells from poly I:C-treated rats	86	113	7	45
2	EC alone	27	56	0	
	IFN-γ	96	155	45	129
	4-day RT6-depleted T cells from rats treated with poly I:C	95	143	16	103
	Con A plus 4-day RT6-depleted T cells from rats treated with poly I:C	92	133	34	127

[a] Data from two representative coculture experiments, each of which used a single EC preparation. Each of the experiments has been repeated three to four times with qualitatively similar results. Rat EC were cultured with medium alone, with rat rIFN-γ (100 U/ml), or with purified DR rat T cells (~20:1 T cell:EC ratio) with or without Con A (3 µg/ml). T cells were isolated from VAF animals that had been treated in vivo with anti-RT6.1 mAb DS4.23 and/or poly I:C for 4 days. Untreated VAF animals were used as controls (nondepleted T cell donors). After 96 h of coculture, endothelial MHC Ag expression was determined as described in *Materials and Methods*.
[b] Percentage of EC expressing Ag; mean FI = mean fluorescence intensity per cell (linear scale of 300 channel numbers).

A did not further augment either IFN-γ production or EC MHC expression.

In the final experiment, RT6 depletion was continued for 30 days, at which time all animals had been diabetic for 9 to 10 days. In contrast to the results of 21-day depleted and acute diabetic rats (Table I, experiment 3), RT6⁻ T cells from these chronically diabetic rats had no ability to activate EC (Table I, experiment 4). In coculture these T cells were functionally similar to total T cells from untreated animals. These cells were, however, capable of responding to Con A, indicating that they had not been rendered unresponsive by chronic hyperglycemia (Table I, experiment 4).

To relate these in vitro findings to the onset of insulitis in vivo, pancreata from a sampling of conventionally housed RT6-depleted DR rats were analyzed histologically. No insulitis was detected in any of nine rats treated with anti-RT6.1 mAb for 8 days. In contrast, pancreata from three of six rats treated for 10 days contained focally inflamed islets. These findings are in agreement with previous reports of DR rats depleted of RT6⁺ T cells in an identical manner (21).

Depletion of RT6⁺ T cells without induction of IDDM fails to generate RT6⁻ T cells capable of activating EC

The data in Table I demonstrate that not all RT6⁻ T cell populations activate EC in the absence of mitogen. To characterize RT6⁻ populations further, additional experiments were performed based on our ability to manipulate the frequency of diabetes in RT6-depleted DR rats by choosing animals of different serologic status and age.

In contrast to conventionally housed DR rats, VAF DR rats depleted of RT6⁺ T cells do not become diabetic unless poly I:C is given concurrently (19). We therefore cocultured EC with T cells from VAF DR rats that were untreated, treated with both poly I:C and anti-RT6 mAb, or with each reagent alone. The results are shown in Table II.

We observed that RT6⁻ T cells from VAF animals treated with both DS4.23 mAb and poly I:C activate EC in the absence of Con A (Table II, experiments 1 and 2). RT6⁻ T cells from nondiabetic VAF animals treated with both reagents for 4 days (Table II, experiments 1 and 2) were functionally similar to RT6⁻ T cells from conventionally housed animals treated with antibody alone for 4 days (Table I, experiment 2). In both instances, the RT6⁻ T cell populations induced maximal class I and submaximal class II expression on EC, and Con A treatment further augmented class II induction. In contrast, VAF DR rats treated for 4 days with either DS4.23 mAb or poly I:C alone yielded T cell populations that did not induce endothelial class II MHC expression in the absence of exogenous Con A (Table II, experiment 1). Treatment with either reagent alone for up to 18 days also failed to produce T cell populations capable of activating EC (data not shown).

We next studied T cells from DR rats depleted of RT6⁺ T cells beginning at >120 days of age (Table III). As is the case with VAF DR rats, RT6 T cell depletion of older, conventionally housed DR rats does not induce IDDM (15). RT6⁻ T cells isolated from older nondiabetic DR rats treated with DS4.23 mAb for 5 to 7 days were functionally similar to total T cells isolated from untreated animals. Neither T cell population induced class II MHC expression by EC.

Table III
Endothelial MHC expression (% positive cells and mean
fluorescence intensity) after coculture with total T cells
and RT6⁻ T cells purified from DR rats >120 days of age[a]

Expt.	Endothelial Cell Coculture Conditions	Class I MHC		Class II MHC	
		%[b]	Mean FI	%	Mean FI
1	EC alone	17	40	0	
	IFN-γ	96	122	41	127
	Total T cells	17	40	0	
	RT6⁻ T cells	21	44	0	
2	EC alone	15	55	0	
	IFN-γ	96	160	81	176
	Total T cells	15	69	0	
	RT6⁻ T cells	35	83	0	

[a] Data from two representative coculture experiments from a series of three
experiments, each of which used a single EC preparation. Rat EC were cultured
with medium alone, with rat rIFN-γ (100 U/ml), or with purified DR rat T cells
(~20:1 T cell:EC ratio). T cells were isolated from conventionally housed
animals that had received no treatments (total T cells), or were treated in vivo
with anti-RT6.1 mAb DS4.23 (RT6⁻ T cells) for 5 days (experiment 1) or 7 days
(experiment 2). After 96 h of coculture, endothelial MHC Ag expression was
determined as described in Materials and Methods.
[b] Percentage of EC expressing Ag; mean FI = mean fluorescence intensity
per cell (linear scale of 300 channel numbers).

RT6⁻ T cells purified by in vitro panning do not activate EC

The data shown in Tables I to III suggest that the differential ability of total T cells vs RT6⁻ T cells to activate EC depends on the conditions under which RT6⁻ cells are generated. Specifically, in vivo conditions that lead to diabetes appear to be required. To explore this concept further, RT6⁻ and RT6⁺ rat T cell populations were prepared by in vitro panning from total LN T cells (Table IV).

In vitro purified RT6⁻ and RT6⁺ T cells were both functionally similar to total T cells (Table IV, experiment 1). All three populations induced only small increases in EC class I MHC expression and no de novo class II expression. Addition of Con A to cocultures using total T cells or RT6⁻ T cells induced near maximal EC activation (Table IV, experiment 2). Similar results were obtained using both VAF and conventionally housed animals.

We also tested the possibility that total T cells failed to activate EC due to the presence of IFN-inhibitory factors (10), perhaps produced by suppressor RT6⁺ T cells. Resting total LN T lymphocytes were cocultured with EC in the presence of exogenous rIFN-γ. No inhibitory effect was observed relative to IFN-γ alone (Table 4, Exp. 1).

T cell IFN-γ production is stimulated by coculture with EC

Table I demonstrates that T cell IFN-γ production and ability to activate EC covary. This is not surprising, given the ability of IFN-γ to induce MHC Ag (24). The data in Table I do not, however, indicate whether EC are simply the passive target of activated T cell-derived cytokines or

Table IV
Endothelial MHC expression (% positive cells and mean
fluorescence intensity) after coculture with total, RT6⁻
and RT6⁺ T cells purified by in vitro panning[a]

Expt.	Endothelial Cell Coculture Conditions	Class I MHC		Class II MHC	
		%[b]	Mean	%	Mean
1	EC alone	19	65	0	
	IFN-γ	89	108	87	119
	Total T cells	28	88	1	109
	Total T cells + IFN-γ	88	106	94	135
	RT6⁺ T cells	23	87	1	110
	RT6⁻ T cells	33	89	1	107
2	EC alone	10	37	0	
	IFN-γ	99	133	84	156
	Total T cells	53	63	0	
	Total T cells + Con A	84	107	75	141
	RT6⁻ T cells	65	78	1	45
	RT6⁻ T cells + Con A	86	109	63	130

[a] Data from two representative coculture experiments from a series of three
experiments, each of which used a single EC preparation. Rat EC were cultured
with medium alone, with rat rIFN-γ (200 U/ml), or with purified DR rat T cells
from untreated animals (~20:1 T cell:EC ratio) with or without added Con A
(3 µg/ml) or IFN-γ (200 U/ml). Conventionally housed animals were used in
experiment 1, and VAF animals in experiment 2. Purified RT6⁺ or RT6⁻ T cells
were isolated from total T cells by in vitro panning. After 96 h of coculture,
endothelial MHC Ag expression was determined as described in Materials and
Methods.
[b] Percentage of EC expressing Ag; mean FI = mean fluorescence intensity
per cell (linear scale of 300 channel numbers).

whether they engage in positive feedback by enhancing T cell lymphokine production.

To investigate this question, RT6⁻ T cells from RT6-depleted conventionally housed DR rats were either 1) cultured in medium alone, 2) cocultured in contact with EC, or 3) cocultured in the same well as EC but physically separated from the monolayers by 0.45-µm microporous membranes (Table V). In these final experiments, the availability of a new anti-rat ICAM-1 reagent enabled us to measure ICAM-1 expression as another index of EC activation. We observed that maximal EC response requires direct contact with T cells. Endothelial MHC Ag and ICAM-1 expression increased but only to submaximal levels in cocultures where T cells were not in direct contact with EC. This suggests that RT6⁻ T cell IFN-γ production occurred but only to a limited extent without T cell-EC contact.

To explore this observation further, IFN-γ concentrations in several coculture supernatants were measured. We first studied RT6⁻ T cells from conventionally housed DR animals treated with anti-RT6 mAb for 18 days (Fig. 2). When cultured in the absence of EC, these T cells produced detectable levels of IFN-γ. When cocultured in the same well as EC, but with direct contact between the two cell types prevented by a microporous membrane, the concentration of IFN-γ increased ~4-fold. In contrast, coculture in direct contact with EC augmented production ~150-fold. In contrast, no IFN-γ could be detected in

Table V
Endothelial ICAM-1 and MHC expression (% positive cells and mean fluorescence intensity) after coculture with RT6⁻ T cells, with and without direct T cell-EC contact[a]

Expt.	Endothelial Cell Coculture Conditions	Class I MHC		Class II MHC		ICAM-1	
		%[b]	Mean FI	%	Mean FI	%	Mean FI
1	EC alone	11	44	0		26	79
	IFN-γ	91	117	78	166	98	72
	8-day depleted T cells without contact	33	73	0		38	90
	8-day depleted T cells with contact	78	119	7	51	56	97
2	EC alone	22	60	1	56	59	116
	18-day depleted T cells without contact	35	93	5	122	67	130
	18-day depleted T cells with contact	92	141	61	145	92	162

[a] Data from two representative coculture experiments, each of which used a single EC preparation. Each of the experiments has been repeated three times with qualitatively similar results. Rat EC were cultured with medium alone, with rat rIFN-γ (300 U/ml), or with purified DR rat T cells (~20:1 T cell:EC ratio). T cells were isolated from conventionally housed animals that had been treated in vivo with anti-RT6.1 mAb DS4.23 for the specified length of time. T cells were allowed to either directly contact EC monolayers, or were prevented from contacting the monolayers by 0.45-μm microporous membrane inserts as described in *Materials and Methods.* After 96 h of coculture, endothelial MHC and ICAM-1 Ag expression was determined.
[b] Percentage of EC expressing Ag; mean FI = mean fluorescence intensity per cell (linear scale of 300 channel numbers).
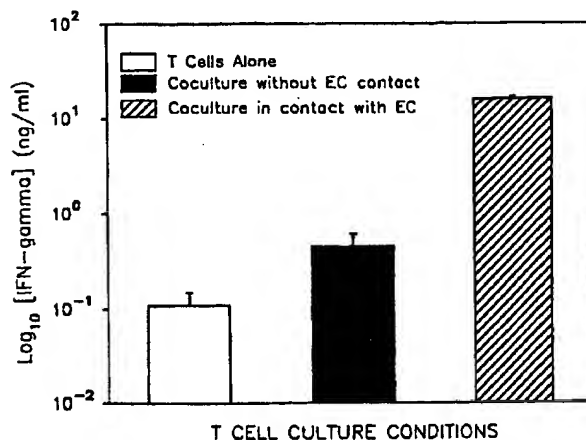
FIGURE 2. IFN-γ production by RT6⁻ T cells following culture in medium alone or coculture with or without direct contact with EC. T cells were isolated from conventionally-housed DR rats in vivo depleted of RT6⁺ T cells for 18 days. T cells were then cultured in (A) medium alone (n = 7), (B) cocultured with EC but separated from the monolayers by 0.45-μm microporous filters (n = 3), or (C) cocultured in direct contact with EC (n = 3). After 96 h, culture supernatants were harvested and IFN-γ levels determined as described in *Materials and Methods.* Data are presented as means ± SEM.

supernatants from cultures carried out using T cells unable to activate EC (Table I, experiments 1 and 4).

Discussion

Our data indicate that RT6⁻ T lymphocytes capable of activating EC are generated during the induction of autoimmune diabetes in the RT6-depleted DR-BB rat. RT6⁻ T cells acquire this capability before the appearance of pancreatic insulitis, and maximal EC stimulating activity is observed at the time of diabetes onset.

RT6 depletion of DR rats for as few as 4 days yields RT6⁻ T cells capable of activating EC. Inasmuch as in-

sulitis did not occur until 10 days of depletion, we hypothesize that in vivo the onset of endothelial activation precedes and may therefore contribute to the onset of inflammation.

RT6⁻ T cells from newly diabetic rats are effective activators of EC; their potency is equivalent to that achieved by optimal concentrations of rIFN-γ. In contrast, RT6⁻ T cells from rats diabetic for >7 days no longer activate EC. This loss of EC-stimulating capability is not due to inherent nonresponsiveness, because RT6⁻ T cells from chronic diabetics maximally activate EC after Con A stimulation. We interpret these findings to suggest that the ability of RT6⁻ T cells to activate EC is not indicative of a permanent phenotypic change, but represents instead a state of transient activation.

The data do not permit us to define precisely the composition of the RT6⁻ cell population with these properties. Previous studies have shown that the DS4.23 antibody neither masks RT6 Ag not modulates it off the cell surface (25), but the occult participation of mature RT6⁺ T cells in this process cannot be excluded for two reasons. First, it is known that rats circulate a population of RT6⁻ T cells destined eventually to become RT6⁺ (17, 26). Second, activation per se is known to modulate RT6 off the cell surface (27). For both reasons, participation of small numbers of RT6⁺ T cells in EC activation cannot be excluded. Our data indicate that the T cell populations used were ≥97% pure, but this does not exclude the participation of small numbers of NK cells in our assays. As noted previously, among the RT6⁻ T cells analyzed before EC coculture, both CD4⁺ and CD8⁺ subpopulations were present. The composition of the residual RT6⁻ cells harvested from antibody treated rats does not, however, alter our basic conclusion that RT6-depletion of DR rats yields an RT6⁻ cell population with the ability to activate EC.

The ability of RT6⁻ cells from DR rats to activate EC

appears to be intimately related to the induction of autoimmune disease. This was established in the experiments that used either VAF or elderly (>120 day old) DR animals. In vivo treatment with RT6-depleting antibody alone did not induce diabetes in these animals, and their RT6⁻ T cell populations were unable to activate EC in the absence of exogenous mitogen. In vitro purified RT6⁻ T cells also failed to activate EC. We conclude that the ability of RT6⁻ T cells to activate EC is not simply a function of the absence of RT6⁺ lymphocytes, but rather is in some way a consequence of the in vivo process that leads to the induction of autoimmunity.

In the DR rat model of IDDM, there appear to be three requirements for the generation of T cells that effectively activate EC. One is the removal of RT6⁺ T cell populations: these are hypothesized to function as suppressor cells. The RT6 Ag is known to mark T cell populations capable of suppressing autoimmune diabetes and thyroiditis (15, 16). The second requirement is some form of immune system activation. This can be either infection with an appropriate virus such as KRV or administration of an activating agent like poly I:C. Both have previously been reported to enhance the induction of autoimmunity in DR rats (19, 20). The third requirement is direct T cell contact with EC. This results in enhanced T cell IFN-γ production and maximal EC activation. We suggest that this final step is a form of endothelial costimulation of T cells, and we conclude that in our system both EC and T cells activate one another. Because the first two requirements outlined above are necessary for the development of autoimmune IDDM in the DR rat, we speculate that direct T cell-EC contact leading to mutual activation may also play a key role in the disease process.

EC supply both soluble (12) and membrane-bound (13, 14) costimulatory signals to T cells. The analysis of T cell IFN-γ production just described leads us to conclude that both soluble and membrane-bound signals are provided to RT6⁻ T cells by EC. The stimulatory effects of the membrane-bound signals are about 30 to 40 times stronger than those provided by soluble factors. The observation of EC activation by RT6⁻¹ T cells in the absence of direct cellular contact also makes it unlikely that our observations are a consequence of allogenic (although MHC identical) interactions between the Wistar Furth EC and DR T cells. It is important to point out that in our study, both signals were supplied by EC that were MHC compatible and initially class II⁻. These characteristics suggest that analogous processes could plausibly occur in vivo; resting EC in animals susceptible to autoimmune disease are likely initially to interact with syngeneic T cells in a non-MHC dependent manner.

It could be argued that total T cells would have activated EC to a maximal degree were it not for the presence of some form of suppression. The RT6⁺ subset of total

T cells is capable of suppressing IDDM after transfusion and engraftment into spontaneously diabetic diabetes prone-BB rats (16). Also, CD4⁺ T cells have been observed to produce IFN-γ-inhibitory factor(s) capable of limiting endothelial class II MHC induction (10). However, when we cocultured resting total LN T cells in the presence of exogenous rIFN-γ, no evidence of inhibition was observed. We believe it unlikely that the failure of total DR rat T cells to activate MHC compatible EC was due to the production of inhibitory signals.

In several of our experiments RT6⁻ T cells were somewhat more effective, and RT6⁺ T cells somewhat less effective, than total T cells as inducers of endothelial class I MHC expression (Table I, experiment 1; Table II, experiment 1; Table III; Table IV). This finding may relate to the differential expression of RT6 on T cell subsets. Purified RT6⁻ T cells are predominantly CD4⁺, because more CD8⁺ than CD4⁺ cells express RT6 (17). We suspect, but cannot prove, that EC activation is due to the CD4⁺ RT6⁻ T cells that predominate in RT6⁻ populations because it has previously been shown that CD4⁺ T cells induce endothelial class I and ICAM-1 expression more effectively than do CD8⁺ T cells (10).

Irrespective of the phenotype of the RT6⁻¹ T cells we studied, the question arises as to why the RT6⁻ LN T cells that we studied were not already fully activated within the lymph nodes. We hypothesize for a number of reasons that few maximally activated RT6⁻ T cells circulate in RT6-depleted DR rats. First, the maximally activated RT6⁻ T cell may not be a stable phenotype; this is suggested by our studies of chronically diabetic rats. Alternatively, as a result of contact with vascular EC in vivo, RT6⁻ T cells may be maximally activated but then emigrate into the pancreas. The demonstration of TNF, IL-1, and IL-6 mRNA expressing T cells in the islets of spontaneously diabetic DP rats supports this concept (28).

One final issue concerns the relationship of our findings to the relatively organ-specific nature of autoimmune inflammation. The BB rat, for example, develops only insulitis and thyroiditis (29, 30). Given previous descriptions of activated pancreatic endothelium in IDDM (3–5), we hypothesize that endothelial activation in IDDM is organ specific, and contributes to organ-specific autoimmune inflammation. What accounts for the organ specificity remains unknown.

In conclusion, our data suggest a relationship between autoimmune diabetes and T cell-mediated endothelial activation. Our in vitro observations support the hypothesis that endothelial activation may be one of the earliest pathologic events in the development of IDDM and may either initiate or enhance subsequent leukocyte infiltration into the pancreas (3). Endothelial activation has been observed in several autoimmune diseases including lupus nephritis (31), thyroiditis (32), experimental encephalitis

(33), experimental myositis (34), and rheumatoid arthritis (35, 36). Our findings may therefore have implications not only for IDDM but for autoimmunity in general.

Acknowledgments

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Reactive oxygen intermediates in autoimmune islet cell destruction of the NOD mouse induced by peritoneal exudate cells (rich in macrophages) but not T cells

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Summary The non-obese diabetic (NOD) mouse spontaneously develops autoimmune Type 1 (insulin-dependent) diabetes mellitus. NOD mice exhibit massive infiltrates of T cells and macrophages into pancreatic islets (insulitis) prior to diabetes. The contribution of oxygen free radicals to the development of insulitis in NOD mice was examined by administration of its scavengers, such as superoxide dismutase and catalase. Bovine superoxide dismutase and catalase were each coupled to polyethylene glycol. The treatment with superoxide dismutase-polyethylene glycol reduced the number of islets with insulitis and increased the undamaged islet tissue, as compared with the control group. The treatment with catalase-polyethylene glycol showed a similar tendency which did not reach significance. Using a flow cytometric assay of the oxidation of 2', 7'-dichlorofluorescein, the content of reactive oxygen intermediates in islet cells in the culture system was measured and the effect of peritoneal exudate cells and T cells on their production examined. Peritoneal exudate cells, but not T cells, from NOD mice increased the content of reactive oxygen intermediates in islet cells of either the NOD mouse or the ILI mouse (MHC-identical to NOD); the addition of superoxide dismutase to the culture medium suppressed this increase in NOD or ILI islet cells. The present data support the concept that production of oxygen free radicals mediated by macrophages can damage islet beta cells, directly resulting in autoimmune Type 1 diabetes in NOD mice. [Diabetologia (1994) 37: 22–31]

Key words NOD mice, insulitis, reactive oxygen intermediates, superoxide dismutase, peritoneal macrophages.

Lymphoid cell infiltration into pancreatic islets (insulitis) is a well-recognized feature of Type 1 (insulin-dependent) diabetes mellitus both in humans [1] and in rodents with spontaneous diabetes [2, 3]. The non-obese diabetic (NOD) mouse spontaneously develops an insulin-dependent diabetes. NOD mice exhibit massive infiltrates of lymphoid cells and macrophages into pancreatic islets (insulitis) prior to complete beta-cell destruction, and insulitis appears as early as 5 weeks of age. Makino and co-workers [4] have shown

that T cells play an important role in the development of insulitis and diabetes in nude NOD mice. The major infiltrating cell populations in the insulitis lesion are CD4+ (L3T4, helper) and CD8+ (Ly2, cytotoxic/suppressor) T cells, and macrophages [5]. Splenocytes from diabetic NOD mice can transfer diabetes to irradiated non-diabetic young NOD mice [6]. Both CD4+ and CD8+ splenic T cells are required to transfer diabetes from diabetic NOD mice to irradiated non-diabetic NOD mice. In T-cell mediated immune processes, the immune responses to self-antigens are dependent on certain combinations of MHC and the T-cell receptor (TcR) $V\beta$ family. Restricted TcR usage has been found in some T-cell dependent autoimmune disease, for example TcR $V\beta 8$ is preferentially used in experimental autoimmune encephalomyelitis [7]. Restriction of TcR usage in autoimmune diabetes has not

been confirmed in the NOD mouse [8, 9]. However, T cells bearing TcR Vβ8 are the major population in the islet-infiltrating cells of diabetic NOD mice [5]. The precise mechanisms by which lymphocytes and macrophages damage islet beta cells remain unclear.

In chemically-induced diabetes, several mechanisms of islet beta-cell damage have been proposed. Streptozotocin and alloxan break nuclear DNA strands of islet beta cells by generating oxygen free radicals [10, 11]. Streptozotocin and its nitrosoamide moiety methylnitrourea alkylate the DNA of beta cells and cause comparable amounts of DNA strand breakage [12, 13]. Streptozotocin alkylates not only DNA but also other key cellular components [14]. Poly (ADPribose) synthetase inhibitors such as nicotinamide and 3-aminobenzamide can scavenge oxygen free radicals in pancreatic beta cells produced by streptozotocin and alloxan in monolayer culture system [15]. Since nicotinamide and 3-aminobenzamide were also reported to prevent the development of spontaneous diabetes in the NOD mouse [16], the same mechanism may be involved in islet beta-cell destruction of the NOD mouse as that in streptozotocin- and alloxan-induced diabetes. Macrophages [17] and probably T cells produce oxygen free radicals in inflammatory reactions. It is reasonable to hypothesize that oxygen free radicals produced by macrophages and T cells in the insulitis lesion may destroy islet beta cells leading to the development of diabetes in NOD mice [18, 19] and that oxygen free radical scavengers, such as superoxide dismutase (SOD) and catalase, may prevent the destruction of beta cells and ensuing diabetes. In this study, we have examined the effects of SOD and catalase on the development of insulitis, and splenic T-cell populations in NOD mice. In addition, the production of reactive oxygen intermediates in pancreatic islet cells induced by peritoneal exudate cells (rich in macrophages) and T cells from celiac lymph nodes of the NOD mouse in vitro was investigated.

Materials and methods

Animals

Non-obese diabetic (NOD) mice were originally obtained from the colony of Dr. S. Makino at the Aburahi Laboratories, Shionogi and Company, Ltd., (Shiga, Japan). NOD/Shi//Jos females develop diabetes at 92% incidence and NOD males at less than 60% incidence by 30 weeks of age. The Institute of Cancer Research-L-Ishibe (ILI) mouse, which is a sister strain of the NOD mouse and is MHC-identical to the NOD mouse, does not develop insulitis or diabetes [20]. Male and female NOD/Shi//Jos and ILI/Jic//Jos mice at 5 months of age were used as a source of pancreatic islet cells in this study. Diabetic NOD mice within 2 weeks after the onset of diabetes were used as a source of peritoneal exudate cells. For the assay of oxygen free radical production in islet cells by NOD peritoneal exudate cells, sex-matched animals were used. The NOD mice were given ad libitum commercial chow (Agway Prolab 3000 Formula; Agway, Syracuse, NY, USA), and water.

Treatment with superoxide dismutase-polyethylene glycol and catalase-polyethylene glycol

Fifteen NOD female litter mates from three litters were divided into three groups at 10 weeks of age. Five female NOD mice in each group were injected (intraperitoneally, daily for 14 days) with either superoxide dismutase-polyethylene glycol (SOD-PEG, 225 U per day per mouse, 55 µg protein, source: bovine erythrocytes; Sigma, St. Louis, Mo, USA), catalase-PEG (2,310 U per day per mouse, 57 µg protein, source: bovine liver; Sigma), or the vehicle alone (PEG, 45 µg per day per mouse; Sigma). SOD-PEG, catalase-PEG and PEG were dissolved in phosphate-buffered saline (PBS; 137 mmol NaCl, 15 mmol Na_2HPO_4, 1.47 mmol KH_2PO_4, 2.68 mmol KCL), pH 7.3. For PEG alone 45 µg was an equivalent dose compared to the compound of SOD-PEG (225 U). The animals were killed at 12 weeks of age after collecting blood samples under ether anaesthesia. The pancreas and spleen were removed and subjected to histological and immunological examinations, respectively.

Assay of the activities of SOD and catalase in plasma

Blood samples (0.4–0.5 ml) were collected with a heparinized pasteur pipette from the retro-orbital venous plexus of the animals under ether anaesthesia. Each animal was assayed for SOD and catalase before and after the treatment. The activity of SOD in heparinized plasma was measured according to the method of McCord and Fridovich [21]. Plasma was diluted ten-fold with 0.9% NaCl (in catalase-PEG and control group) or 100-fold (SOD-PEG group) and used for the SOD assay. The activity of catalase in plasma was assayed according to the method of Beers and Sizer [22]. One unit of catalase decomposed 1.0 µmol hydrogen peroxide per min at 25°C.

Point-counting morphometrics of the islets

The animals were killed under ether anaesthesia, and pancreases removed and weighed. For morphometric analysis it has been shown that there is usually more biological variation between animals than between sections if the tissue is either homogenous or consistently oriented in the same plane. The pancreases were oriented in the same plane and spread prior to fixation in Bouin's solution. With these procedures, a large cross-sectional area of pancreas was obtained and only one "level" or section per animal was required to have adequate number of intercepts (5000 points per animal). The tissues were embedded in paraffin, cut at 6 µm and stained with haematoxylin and eosin. Using one section per animal, all islets on each section were scored for insulitis; the number of islets per section ranged from 17 to 41. Two separate quantifications were performed to evaluate the protective effect of SOD and catalase on islet cell destruction. First, the number of islets with insulitis and the total number of islets were counted at 100 × magnification. The ratio of these values multiplied by 100 gave the percent islets with insulitis. Secondly, the islet portion without infiltrating immune cells was defined as the undamaged islet portion. The relative volume of undamaged islet portion was measured by point-counting morphometrics [23] at 170 × magnification, covering the entire section with non-overlapping fields of a 50-point grid. The number of points counted per animal was approximately 5,000. The absolute mass of undamaged islet portion was then calculated by multiplying the relative volume by the pancreatic weight for each animal.

Antibodies

Monoclonal antibodies (mAb) used to characterize splenic lymphoid cells and peritoneal exudate cells were fluorescein isothionate (FITC)-conjugated or biotinylated mAb anti-mouse Thy1.2 (Becton Dickinson, Mountain View, Calif., USA) for T cells, phycoerythrin (PE)-conjugated mAb GK1.5 anti-L3T4 (Becton Dickinson) for CD4 T cells, FITC-conjugated mAb 53-6 anti-mouse Ly2 (Becton Dickinson) for CD8 T cells, FITC-conjugated goat anti-mouse IgM (Organon Teknika, Malvern, Pa., USA) for B cells, mAb M3/84.6.34 (rat IgG1, American Type Culture Collection, Rockville, Md., USA) and mAb M1/70.15 (rat IgG2b, Caltag Laboratories, South San Francisco, Calif., USA) for macrophages, mAb PK 136 anti-mouse natural killer cells (IgG2a) and rabbit anti-asialo GM1 (Wako Fine Chemical, Dallas, Tx., USA) for natural killer cells. Antisera used as controls were: monoclonal antibody P3X63 for mAb anti-mouse Thy1.2, normal mouse serum for anti-mouse IgM, mAb anti-L3T4 and Ly2, normal rat IgG for mAb M3/84.6.34 and mAb M1/70.15, and normal rabbit serum for rabbit anti-asialo GM1.

Monoclonal antibodies used in studying TcRαβ, TcR Vβ8 and CD3 (ε) were mAb H57-597 (hamster IgG) pan anti-mouse TcRαβ [24], mAb F23.1 (mouse IgG 2a) anti TcR Vβ8.1, 2, 3 [25], and mAb 145-2C11 hamster anti-mouse CD3 (ε) [26], respectively. Antisera used as controls were: normal hamster serum for mAb 57-597.2 and 145-2C11, and purified mouse myeloma RPC 5.4 (IgG 2a, Organon Teknika) for mAb F23.1.

Splenic T cells

Spleens were dissected from the 12-week-old NOD mice after the 2-week administration of SOD-PEG, catalase-PEG and PEG, and dissociated by gentle passage through a wire screen. Lymphoid cells were separated from erythrocytes with Ficoll-400 (Lympholyte-M; Cederlane, Province, Canada), and washed twice with RPMI-1640 medium containing 5% fetal calf serum (FCS). B cells were stained directly with FITC-conjugated goat anti-mouse IgM. The lymphoid cell suspensions were applied to a nylon wool column (Wako Fine Chemical), and incubated at 37°C for 45 min. T cells were then eluted with pre-warmed RPMI-1640 medium containing 5% FCS. T cells were stained with FITC-conjugated mAb anti-mouse Thy1.2 before and after the purification on nylon wool column to determine the recovery and the purity of T cells (95%).

The purified T cells were incubated with FITC-conjugated mAb anti-mouse Ly2 or phycoerythrin-conjugated mAb anti-mouse L3T4 on ice for 45 min, washed three times with medium RPMI-1640. Furthermore, the purified T cells were incubated with mAb 145-2C11 (TcRαβ), mAb H57-597 (TcR Vβ8) or heat-inactivated normal hamster serum on ice for 45 min. T cells were incubated with mAb F23.1 or mouse myeloma protein RPC 5.4 at 37°C for 30 min. After the incubation, T cells were washed three times with RPMI-1640 medium, and then stained with FITC-conjugated goat anti-hamster IgG or goat anti-mouse IgG (Fc specific). The cells were fixed in 2% formaldehyde-PBS, and stored at 4°C until flow cytometric analysis. The percentage of fluorescein-positive cells were measured by flow cytometer. The absolute number of B cells and T cells in each subset was estimated from the total number of cells and the percentage of positive cells.

Islet cell preparations

Islets were isolated from non-diabetic 16-week-old NOD or ILI males. NOD males were chosen because of the lesser degree of islet damage than in NOD females. Islets were also isolated from

ILI males. ILI mice were chosen because this strain is MHC-identical to the NOD mouse and develops neither insulitis nor diabetes. The animals were anaesthetized intraperitoneally with pentobarbital (Na Salt; 0.5–1.0 mg/mouse; Sigma). Collagenase Type IV (Organon Teknika) solution (4 ml of 2 mg/ml in Hank's balanced salt solution) was infused into the pancreatic duct after ligating the common bile duct at its entrance to the duodenum. The expanded pancreas was digested at 38°C for 30 min and islets were purified on Ficoll gradients (25, 23, 20.5 and 11%) and centrifuged at room temperature (×800 g, 10 min). The islets were hand picked to avoid contamination by exocrine cells and connective tissues. To prepare single islet cells, isolated islets were washed with Ca- or Mg-free PBS and digested with trypsin solution for 2 min. After complete dissociation, the islet cells were washed twice with medium RPMI-1640 containing 5% FCS.

Peritoneal exudate cells

Peritoneal exudate cells were collected from non-diabetic 16-week-old NOD males in Experiment 1 and diabetic NOD females in Experiment 2 by lavage of the peritoneal cavity with RPMI-1640 medium containing 2% FCS. The peritoneal exudate cells consisted of macrophages (60%), T cells (7%), B cells (6%) and anti-asialo GM1$^+$ cells (27%) in non-diabetic NOD males and diabetic NOD females shortly after the onset of diabetes. Peritoneal T cells (PTc) were prepared by passing the peritoneal exudate cells through nylon wool columns (T-cell purity: 97%). The macrophage-enriched fraction (peritoneal macrophages, PMø) was recovered from the nylon wool (PMø purity: 75%) and contained a very small number of T cells (less than 3%).

Celiac lymph node T cells

Lymphoid cells from the pancreas are mainly drained into the celiac lymph nodes and through the efferent lymphatic vessels leading to the cisterna chyli. Diabetic and non-diabetic NOD mice but not C57BL/6J, BALB/cJ and NON/Jos mice showed enlargement of the celiac lymph nodes with an increased number of T cells (unpublished observation). Injection of lymphocytes (2 × 10^7) from the enlarged celiac lymph nodes from diabetic NOD mice transferred diabetes into irradiated young NOD mice. As a source of T cells, celiac lymph nodes were dissected from non-diabetic 16-week-old NOD males or diabetic NOD females, and dissociated to yield single-cell suspensions. The cell suspensions contained T cells (70%), B cells (26%) and macrophages (6%) and T cells were purified by passing through a nylon wool column.

Measurement of reactive oxygen intermediates (hydrogen peroxide) in islet cells

Four experiments were designed to investigate the production of reactive oxygen intermediates in islet cells. Experiment 1 was designed to test the production of reactive oxygen intermediates in islet cells from male NOD or ILI mice with peritoneal exudate cells and T cells from non-diabetic NOD males. Islet cells isolated from either NOD males (culture A–E) or ILI males (culture F–J) were used for the measurement of reactive oxygen intermediates. Peritoneal exudate cells were prepared from the non-diabetic NOD male mice. Isolated islet cells (2 × 10^5 cells), celiac T cells (nylon wool purified T cells, 4 × 10^5 cells), and peritoneal exudate cells (4 × 10^5 cells) were cultured overnight in the
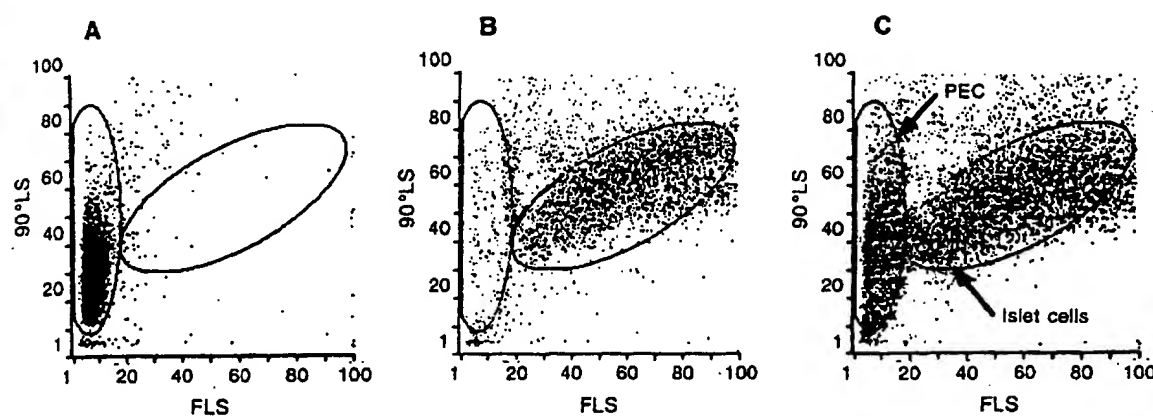
Fig. 1A–C. Cytograms of bit map gating for distinguishing (A) peritoneal exudate cells (PEC), (B) islet cells and (C) both PEC + islet cells from NOD mice. Five thousand PEC and 5000 islet cells were examined by light scatter characteristic. FLS, forward light scatter; 90° LS, 90° light scatter

medium RPMI-1640 (total volume, 200 µl) containing 5% FCS, 15% concanavalin-A-stimulated NOD splenocyte conditioned media, and 1% fresh L-glutamine in a humidified atmosphere (5% CO_2 and 95% air, at 37°C). Several combinations of islet cells, T cells and peritoneal exudate cells were tested in the culture system. After overnight culture, the content of reactive oxygen intermediates (hydrogen peroxide) in islet cells was measured using the flow cytometric method of Bass and co-workers [27]. The 2′, 7′-dichlorofluorescein diacetate (DCFH-DA; Eastman Kodak, Rochester, NY, USA) was added to the medium (final concentration, 50 µmol/l), and cells were then cultured for 20 min at 37°C. DCFH incorporated into islet cells was oxidized rapidly to fluorescent form by intracellular hydrogen peroxide. SOD was added to culture E or J (final concentration, 13.4 U/ml) in combination with DCFH-DA. Then, the fluorescence of islet cells was determined on a flow cytometer (Cytoflurorograph 50H; Ortho Diagnostic System Inc., Westwood, Mass., USA). Islet cells were separated from the co-culturing peritoneal exudate cells (PEC) by electric gating on a forward light scatter vs 90° bit map (Fig. 1).

Experiment 2 was designed to test the production of reactive oxygen intermediates in islet cells from female ILI mice with peritoneal exudate cells and T cells from diabetic female NOD mice. Islets were isolated from non-diabetic 16-week-old ILI females. Peritoneal exudate cells and celiac lymph node cells were prepared from diabetic NOD females within 2 weeks after the onset of diabetes. The ILI islet cells were cultured with peritoneal exudate cells, peritoneal macrophages, peritoneal T cells or T cells of the celiac lymph nodes from the diabetic NOD females. The culture conditions were the same as in Experiment 1 except that the culture time of the islet cells with peritoneal exudate cells was reduced from overnight to 3 h. Reactive oxygen intermediate production in the islet cells was measured in the same manner as in Experiment 1.

Experiment 3 was designed to test direct oxidation of 2′, 7′-dichlorofluorescein (DCFH) in islet cells by hydrogen peroxide (H_2O_2). Islet cells (2×10^5) from C3H/HeJ mice (MHC-haplotype: k) were incubated with 2′, 7′-dichlorofluorescein diacetate (final concentration, 50 µmol/l) for 30 min and followed by incubation with hydrogen peroxide (10 to 100 µmol/l; Sigma) for 15 min. The fluorescence of islet cells was then determined on a

flow cytometer. The specificity of peritoneal exudate cells from NOD, ILI and C3H/He mice was also examined.

Experiment 4 was designed to test the production of reactive oxygen intermediates in islet cells by cytokines produced by macrophages. Islet cells (2×10^5) from ILI mice were incubated with recombinant mouse interleukin-1-α (40 U; Genzyme Boston, Mass., USA) for 3 h and followed by incubation with DCFH-DA for 20 min and flow cytometric analysis.

Statistical analysis

Statistical analysis was performed by the Mann-Whitney U test.

Results

Treatment with SOD-PEG or catalase-PEG

The 2-week treatment with SOD-PEG or catalase-PEG did not affect the growth nor cause signs of visible allergic reactions such as skin rash and anaphylaxis in the animals. The plasma activity of SOD in the SOD-PEG group increased more than 100-fold compared with that in the control group (Table 1). The plasma activity of catalase in the catalase-PEG group increased four-fold compared to that in the control group.

The pancreases from the above three groups were subjected to morphometric analysis. The number of islets examined on each section is listed in Table 2. There were no significant changes in the pancreatic weight among the SOD-PEG, catalase-PEG and PEG groups (213.9 ± 8.0 mg, 204.5 ± 8.8 mg, and 196.2 ± 14.2 mg, respectively). The percentage of islets with insulitis was reduced in the SOD-PEG group to 50% of the PEG group and in the catalase-PEG group to 60% of the PEG group (Table 2). With either SOD-PEG or catalase-PEG treatment, the absolute mass of undamaged islets was two times greater than that of the PEG group. Without administration of SOD-PEG, catalase-PEG or PEG, in 3-month-old female NOD mice ($n = 6$) the mean percentage of islets with insulitis was 51.2 ± 9.1%, the relative volume of undamaged islets 0.45 ± 0.10%, and the absolute mass of undamaged is-

Table 1. The plasma activity of superoxide dismutase (SOD) and catalase in NOD mice before and after the 2-week administration of SOD-PEG and catalase-PEG

	Body weight (g)		SOD activity (U/ml)		Catalase activity (U/ml)	
	10 weeks old before	12 weeks old after	before	after	before	after
SOD-PEG	21.6 ± 0.6	22.6 ± 0.9	3.3 ± 0.1	532 ± 17[a]	16.6 ± 1.2	27.2 ± 1.3
Catalase-PEG	22.2 ± 0.2	23.0 ± 0.1	3.0 ± 0.4	2.2 ± 0.1	17.8 ± 1.0	83.0 ± 13.5[a]
PEG alone	21.5 ± 0.7	22.1 ± 0.7	3.6 ± 0.3	3.5 ± 1.2	15.0 ± 2.5	22.2 ± 1.9

Values are expressed as mean ± SEM, n = 5 mice in each group. [a] $p < 0.05$. significant difference from the value of control (PEG alone) in each experiment by Mann-Whitney U test. PEG, Polyethylene glycol

Table 2. Effects of superoxide dismutase-polyethylene glycol (SOD-PEG) and catalase-PEG on the degree of islet damage of the female NOD mouse at 3 months of age

Group	No. of animals	No. of islets/section observed	% of islets with insulitis	Relative volume of un-damaged islet portion (%)	Absolute mass of un-damaged islet portion (mg)
SOD-PEG	5	34 ± 3 (26–41)	18.7 ± 4.8[a] (6.9–30.8)	0.91 ± 0.04[a] (0.79–1.03)	1.93 ± 0.04[a] (1.83–2.06)
Catalase-PEG	5	25 ± 4 (17–34)	25.7 ± 10.5 (4.0–54.2)	0.83 ± 0.16 (0.36–1.27)	1.68 ± 0.30[a] (0.76–2.60)
PEG alone	5	24 ± 4 (17–38)	45.2 ± 5.2 (31.6–57.1)	0.45 ± 0.05 (0.36–0.57)	0.86 ± 0.09 (0.66–1.14)

Values are expressed as mean ± SEM. Number in parentheses is the range.
[a] $p < 0.05$, significant difference from the value of control (PEG alone) in each experiment by Mann-Whitney U test

Table 3. Quantitative analysis of B cells and T cells in the spleen of the NOD mouse after administration of superoxide dismutase-polyethylene glycol (SOD-PEG) and catalase-PEG

	B cells	T cells				
		CD3+ (145–2C11)	CD4+ (GK 1.5)	CD8+ (53-6)	TcRαβ+ (H57-597)	TcR Vβ8+ (F23.1)
SOD-PEG	19.4 ± 3.6	22.1 ± 0.8	17.9 ± 1.1	8.8 ± 1.2	21.9 ± 1.5	3.9 ± 0.5
Catalase-PEG	15.1 ± 1.1	23.8 ± 1.8	19.1 ± 1.7	7.0 ± 0.9	24.8 ± 1.8	2.9 ± 0.3
PEG alone	15.7 ± 1.3	24.2 ± 1.0	18.9 ± 2.1	6.8 ± 0.8	24.7 ± 1.1	2.9 ± 0.9

Values are expressed as mean ± SEM ($\times 10^{-6}$ cells), n = 5 mice in each group. For T cells, monoclonal antibodies in parentheses

lets 0.82 ± 0.18 mg. The degree of islet damages in the untreated NOD females was similar to that in the PEG-treated NOD females at 3 months of age.

The number of splenic B cells and T-cell subsets was examined in the SOD-PEG, catalase-PEG and PEG animals (Table 3). There were no changes in the number of B cells, T cells, CD4+ T cells and CD8+ T cells after the treatment with SOD-PEG or catalase-PEG, nor in the T-cell subsets bearing TcRαβ chains, and TcR Vβ8.1, 2, 3, as compared with those of the PEG alone control group.

Reactive oxygen intermediate production in islet cells

Experiment 1: peritoneal exudate cells from non-diabetic NOD males. Histograms (expanded log scale-X axis) in Figure 2 show fluorescence distribution of DCFH oxidation in islet cells of the NOD mouse and the ILI mouse. Isolated islet cells of the NOD mouse did not produce reactive oxygen intermediates (Fig. 2 A). The T cells from celiac lymph nodes of the NOD mouse did not induce production of reactive oxygen intermediates in the NOD islet cells (Fig. 2 B). Peritoneal exudate cells of the NOD mouse resulted in the production of reactive oxygen intermediates in the NOD islet cells (Fig. 2 C). Combination of peritoneal exudate cells (4×10^5 cells) and T cells (4×10^5 cells) did not enhance and rather slightly suppressed the production of reactive oxygen intermediates in the NOD islet cells (Fig. 2 D). Addition of SOD (13.4 U/ml) to peritoneal exudate cells + T cells partially inhibited the production of reactive oxygen intermediates in the NOD islet cells (Fig. 2 E). Nylon wool (and Sephadex G-10) purified T cells (purity: > 95 %) isolated from the NOD peritoneal exudate cells failed to induce the production of reactive oxygen intermediates in the islet cells.
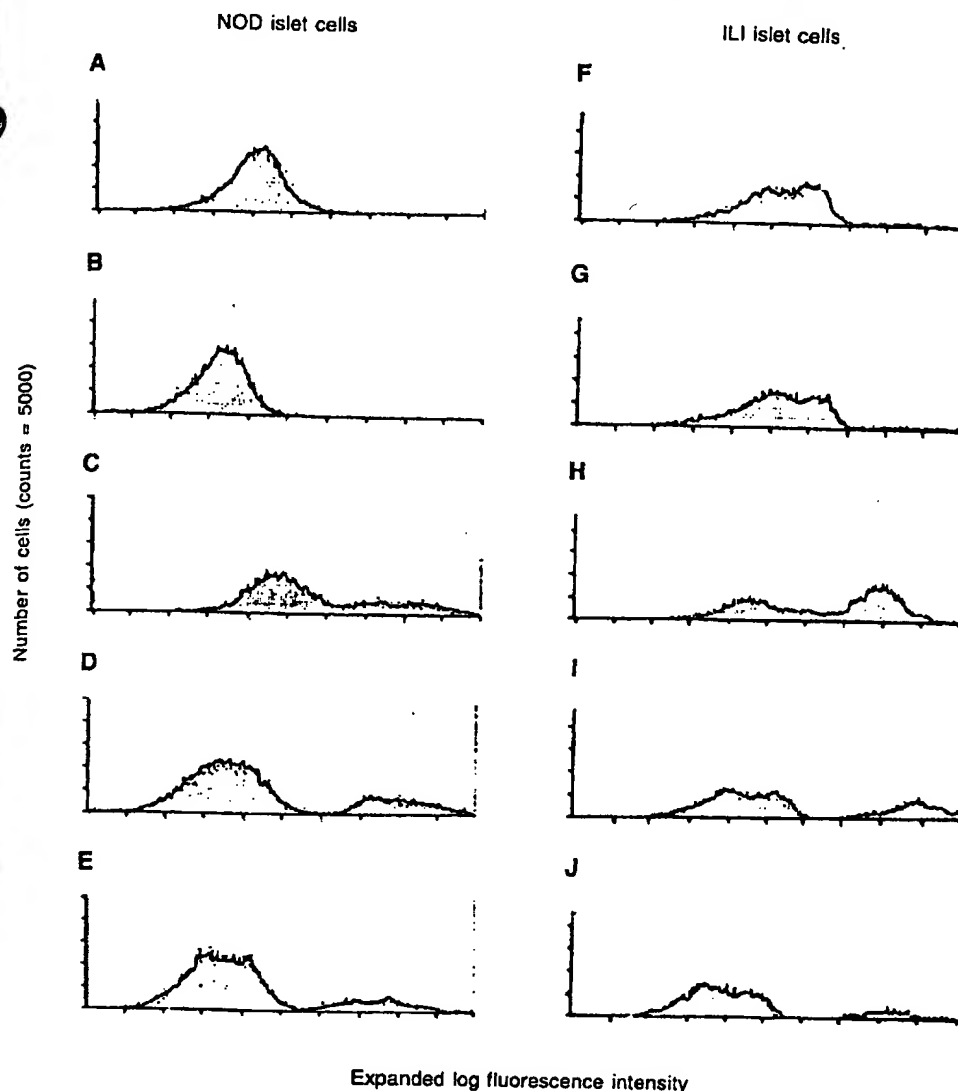
NOD islet cells

A

B

C

D

E

ILI islet cells

F

G

H

I

J

Number of cells (counts = 5000)

Expanded log fluorescence intensity

Fig. 2A–J. Histograms of fluorescence distribution of 2′, 7′-Dichlorofluorescein (DCFH) oxidation in islet cells from NOD or ILI male mice induced by peritoneal exudate cells from non-diabetic NOD male mice. T cells of the celiac lymph nodes from non-diabetic male NOD mice were also tested to induce reactive oxygen intermediates in the islet cells. The islet cells and the effector cells were co-cultured overnight prior to the addition of DCFH-DA. Note the fluorescence intensity (on the X-axis) is expressed as "expanded log scale". Culture conditions were described in Materials and methods. The histograms represent numbers of cells (on the Y-axis) as a function of fluorescence intensity (on the X-axis). Percent of fluorescence-positive islet cells of the NOD mouse is shown in parentheses:
A: NOD islet cells alone (1.2%);
B: NOD islet cells + T cells (0.4%);
C: NOD islet cells + peritoneal exudate cells (35.1%);
D: NOD islet cells + peritoneal exudate cells + T-cells (28.0%);
E: NOD islet cells + peritoneal exudate cells + T cells + superoxide dismutase (19.1%);
F: ILI islet cells alone (7.1%);
G: ILI islet cells + T cells (10.2%);
H: ILI islet cells + peritoneal exudate cells (52.6%);
I: ILI islet cells + peritoneal exudate cells + T cells (44.7%);
J: ILI islet cells + peritoneal exudate cells + T cells + superoxide dismutase, (21.0%). The histograms represent one of five experiments

The ILI mouse is MHC-identical to the NOD mouse but does not develop insulitis or diabetes. Islet cells of the ILI mouse were tested for reactive oxygen intermediate production by peritoneal exudate cells of the NOD mouse. The results from the flow cytometric analysis of the ILI islet cells were similar to those of the NOD islet cells (Fig. 2F–J). These results indicate that the peritoneal exudate cells (rich in macrophages) but not T cells may induce the production of reactive oxygen intermediates in islet cells of the NOD and ILI mice. This production of reactive oxygen intermediates was partially blocked by SOD.

Experiment 2: peritoneal exudate cells from diabetic NOD females. Reactive oxygen intermediates were produced in islet cells from ILI females by peritoneal exudate cells from diabetic NOD females and the suppressive effect of SOD on the production of reactive oxygen intermediates is shown in Figure 3. The islet cells

(2×10^5) were incubated with peritoneal exudate cells (5×10^5). The islet cells showed only 0.3% of fluorescein positive cells by themselves, 51.3% in the presence of peritoneal exudate cells, and 14.0% in the presence of peritoneal exudate cells and SOD (final concentration 13.4 U/ml). The suppressive effect of SOD on the production of reactive oxygen intermediates in ILI islet cells by diabetic NOD females from assays in triplicate is shown in Figure 4. A doubled number of peritoneal exudate cells (10×10^5) slightly increased reactive oxygen intermediates in the islet cells. However, the production of reactive oxygen intermediates was suppressed by peritoneal exudate cells from SOD in both sets of samples.

Reactive oxygen intermediates were produced in islet cells from ILI females by macrophages but not T cells from diabetic NOD females (Fig. 5). Macrophages but not T cells from the diabetic NOD mice induced the production of reactive oxygen intermediates in the ILI islet cells.
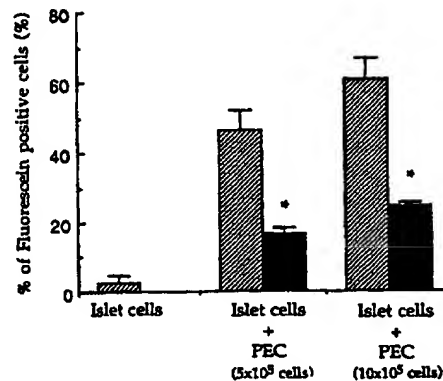
Fig. 4. Suppressive effect of superoxide dismutase (14.3 U/ml) on reactive oxygen intermediate production in ILI islet cells by peritoneal exudate cells from diabetic NOD females. Peritoneal exudate cells (PEC) were tested at 5×10^5 and 10×10^5 cells with 2×10^5 islet cells. Values are expressed as mean ± SEM from assays in triplicate. (▨) SOD-free, (■) SOD-present. *$p < 0.05$ vs SOD-free
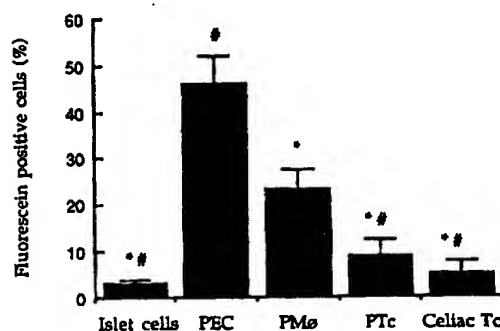

Fig. 5. Reactive oxygen intermediate production in islet cells from ILI females induced by macrophages but not T cells from diabetic NOD females. Islet cells from ILI females (2×10^5 cells) were cultured with peritoneal exudate cells (PEC, 4×10^5; macrophage purity: 60 %), peritoneal macrophages (PMø, 4×10^5; macrophage purity: 75 %), peritoneal T cells (PTc, 4×10^5, T-cell purity: 97 %), or T cells from celiac lymph nodes (celiac Tc, 4×10^5, T-cell purity: 95 %) from diabetic NOD females. Macrophages but not T cells from diabetic NOD females could induce the production of reactive oxygen intermediates in islet cells from ILI females. The bars indicate means ± SEM (%) from five experiments. *$p < 0.05$ vs PEC, # $p < 0.05$ vs PMø

Log fluorescence intensity

Fig. 3 A–C. Suppressive effect of superoxide dismutase (SOD) on reactive oxygen intermediate production by peritoneal exudate cells from diabetic NOD mice. Islet cells were prepared from ILI females and peritoneal exudate cells from diabetic NOD females. The islet cells and peritoneal exudate cells were co-cultured for 3 h prior to the addition of 2', 7'-dichlorofluorescein diacetate. Peritoneal exudate cells from diabetic NOD female mice induced the production of reactive oxygen intermediates even with the ILI islet cells. (**A**) Islet cells only. Reactive oxygen intermediates were produced in islet cells from ILI females by peritoneal exudate cells from diabetic NOD females (**B**) and suppressive effect of superoxide dismutase on the reactive oxygen intermediate production (**C**)

Experiment 3: Cellular oxidation of 2', 7'-dichlorofluorescein in islet cells by hydrogen peroxide (non-specific oxidation). Hydrogen peroxide at 50 μmol/l induced maximal fluorescence-positive islet cells (70.2 %). Hydrogen peroxide at more than 100 μmol/l killed islet cells. To test the specificity of the effects of peritoneal exudate cells from NOD and ILI mice, ILI islet cells were cultured with PEC from NOD or ILI mice. Peritoneal exudate cells from both NOD and ILI could induce reactive oxygen intermediates in the ILI islets. Peritoneal exudate cells from C3H/He, however, could not induce a significant production of reactive oxygen intermediates in islet cells from ILI or C3H/He mice.

Log fluorescence intensity
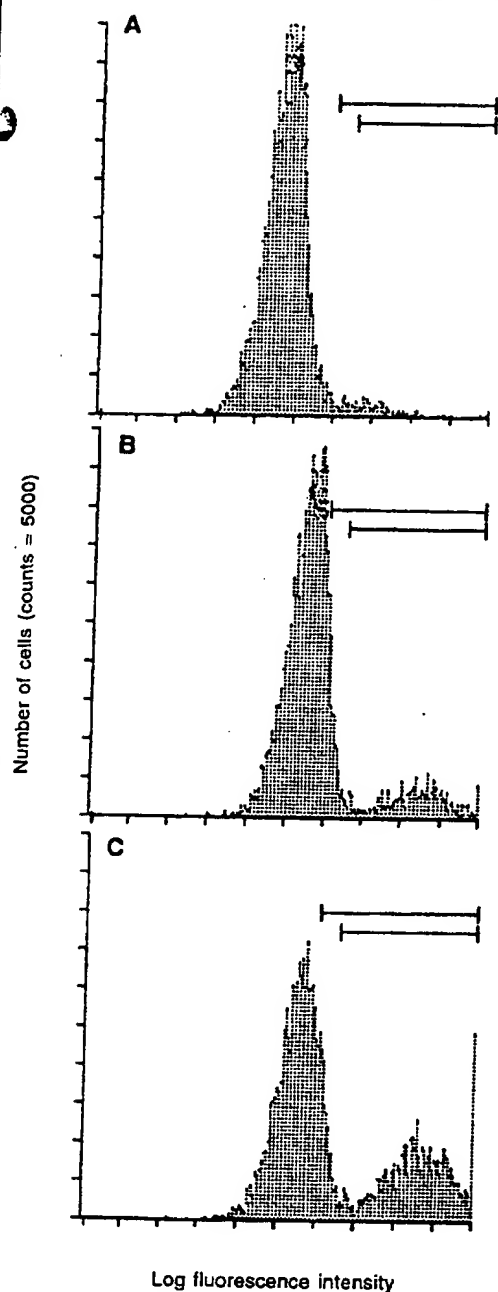
Fig. 6. Reactive oxygen intermediate production in islet cells from ILI females induced by recombinant mouse interleukin-1-α. The islet cells were incubated with recombinant mouse interleukin-1-α (40 U/2 × 10⁵ islet cells) for 3 h prior to the addition of 2′, 7′-dichlorofluorescein diacetate. Percent of fluorescence-positive islet cells of the ILI mouse is shown in parentheses: A: islet cells alone (4.2%); B: islet cells + interleukin-1-α (25.4%); C: islet cells + PEC from diabetic NOD mice (45.9%)

Experiment 4: Reactive oxygen intermediate production by interleukin-1-α. Recombinant mouse interleukin-1-α could produce reactive oxygen intermediates in ILI islet cells (Fig. 6).

Discussion

Pre-treatment with a single intravenous injection of SOD-PEG protects mice against alloxan-induced hyperglycaemia [28]. The treatment with desferrioxamine (inhibitor of hydroxyl radical formation from superoxide anion), nicotinamide, or a combination of desferrioxamine and nicotinamide prevents disease recurrence in diabetic NOD mice after the allograft of BALB/c islets [18]. Nomikos and co-workers [29] have recently reported that SOD and catalase are effective in preventing destruction of islet allografts of the BALB/c mouse in diabetic NOD mice, as measured by continuing ability of the graft to regulate blood glucose.

In the present study, both SOD-PEG and catalase-PEG were effective in preventing islet destruction of the NOD mice. The markedly increased absolute mass of undamaged islet portion observed in the treated groups may be due to the prevention of further beta-cell destruction by the action of SOD and catalase. Meanwhile, SOD and catalase from different species did not cause any allergic reactions, islet beta-cell tumours, or change in body weight in NOD mice. PEG attachment to purified enzymes has been shown to prolong the biological half-life of the enzymes [30]. Therefore, we used SOD-PEG and catalase-PEG instead of SOD or catalase alone. Although PEG itself can suppress immune reactions [31], a comparison of the data from the PEG-treated NOD mice with those from untreated NOD mice indicates that PEG alone had no protective effect on the islet in this study. The degree of islet damage in the untreated NOD females was similar to that in the PEG-treated NOD females at 3 months of age [32]. Therefore, the protection from islet-cell destruction observed in this study seems to be due to SOD and catalase themselves. During the present experiments with SOD-PEG, we had to evaluate its short-term efficacy due to the high cost of SOD-PEG. A long-term (8 month) administration of SOD-PEG and catalase-PEG may be required to see whether the oxygen free radical scavengers coupled to PEG can prevent the development of diabetes in NOD mice.

Injection of splenocytes from diabetic NOD mice can transfer diabetes into irradiated young NOD mice [33]. Both CD4⁺ and CD8⁺ splenic T cells are required for the adoptive transfer of diabetes in NOD mice [6]. Restricted usage of TcR in autoimmune diabetes has not been found in the NOD mouse [8, 9]. However, T cells bearing TcR Vβ8 are the major population in the islet-infiltrating cells of diabetic NOD mice, and that removing a major TcR Vβ family (Vβ8) appears to influence disease progression [5]. In this study, the treatment with either SOD-PEG or catalase-PEG did not change the proportion of B cells, CD3⁺, CD4⁺ and CD8⁺ T cells, T cells bearing TcRαβ chains, nor T cells bearing TcR Vβ8.1,2,3 in the spleen, as compared with those of the control group. Our data suggest that SOD-PEG and catalase-PEG do not exert the preventive ef-

fect on islet beta-cell destruction by changing the number of T cells. In our in vitro studies [34], peritoneal exudate cells (rich in macrophages) or T-cell depleted peritoneal exudate cells (macrophage-enriched fraction) could induce production of reactive oxygen intermediates in the islet cells. However, nylon wool purified peritoneal T cells or T cells from celiac lymph nodes failed to induce production of reactive oxygen intermediates in the islet cells. The effect of peritoneal exudate cells (PEC) appears to be greater than the additive effects of peritoneal macrophages (PMø) and T cells (PTc) despite the increased number of macrophages in PMø fraction. This observation indicates that macrophages may require interaction with T cells for maximal production of reactive oxygen intermediates in islet cells. Furthermore, the production of reactive oxygen intermediates in the islet cells by peritoneal exudate cells was inhibited by SOD-PEG (oxygen free radical scavenger). These observations suggest that SOD-PEG suppressed the production of reactive oxygen intermediates in the islet cells induced by macrophages, and thereby prevented the islet beta-cell destruction in the NOD mice.

Recent studies [35-37] suggest that macrophages play an important role in the initiation of insulitis in NOD mice. We speculate that the role of T cells and macrophages in the development of insulitis is as follows: Initially, an antigen(s) on islet beta cells may be processed by macrophages and presented to CD4+ helper T cells in an MHC class II restricted manner [38]. Indeed, the production of reactive oxygen intermediates in the islet cells by NOD peritoneal exudate cells appears to be MHC class II I-A-restricted [39]. Production of reactive oxygen intermediates in the islet cells was completely blocked by mAb 40A reacting with NOD's MHC class II I-A [40], but not by mAb 14-4-4S reacting with MHC class II I-E, mAb 31-3-4S reacting with MHC class I K^d, or mAb 28-14-8S reacting with class I D^b. After the antigen presentation by the macrophages, the CD4+ helper T cells may be activated by the antigen-presenting cells and secrete lymphokines such as interferon-γ(INF-γ) resulting in activation of macrophages [41]. Activated macrophages may produce and secrete cytokines, such as interleukin-1 (IL-1) [42] and tumour necrosis factor [43], and simultaneously produce oxygen free radicals [11]. It was reported that IL-1 impaired the islet beta-cell activity in vitro [44, 45], and that IL-1, tumour necrosis factor and INF-γ have synergistically cytotoxic effects on islet cells in vitro [46]. In our flow cytometric analysis, recombinant murine IL-1 per se could induce production of reactive oxygen intermediates in the islet cells in vitro. Reactive oxygen intermediates produced either in the islet beta cells by interaction with the cytokines or directly by the activated macrophages could lead to beta-cell destruction.

The data in the present study suggest that in the prevention of islet beta-cell destruction of the NOD

mouse SOD-PEG exerts its action/effect on peritoneal macrophages as effector cells rather than on T cells as antigen recognizing and helper cells. It remains unclear whether the peritoneal macrophages must be stimulated by T-cell derived cytokines to produce reactive oxygen intermediates, or whether the peritoneal macrophages stimulate islet beta cells to produce reactive oxygen intermediates. The present study supports the concept that oxygen free radicals, such as superoxide anion and hydrogen peroxide, damage islet beta cells resulting in the development of diabetes.

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diabetes

PERSPECTIVES IN DIABETES

ORGANIZATION SECTION

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Perspectives in Diabetes

Immunoregulatory and Cytokine Imbalances in the Pathogenesis of IDDM

Therapeutic Intervention by Immunostimulation?

Alex Rabinovitch

The autoimmune response that leads to destruction of pancreatic islet β-cells and insulin-dependent diabetes mellitus (IDDM) has a genetic basis; however, environmental factors can exert profound modulating effects on the genetic predisposition to this autoimmune response. Recent studies in animal models for human IDDM, the genetically diabetes-prone NOD mouse and BB rat, have revealed that microbial agents—including certain viruses and extracts of bacteria, fungi, and mycobacteria—often have a protective action against diabetes development. Many of these microbial preparations are immune adjuvants, which are agents that stimulate the immune system. The protective effects of these agents against diabetes appear to involve perturbations in the production of cytokines, which are polypeptides produced by and acting on cells of the immune system. Thus, recent studies in NOD mice suggest that the islet β-cell–directed autoimmune response may be mediated by a T-helper 1 (Th1) subset of T-cells producing the cytokines interleukin-2 (IL-2) and interferon-γ. These studies also suggest that the diabetes-protective effects of administering microbial agents, adjuvants, and a β-cell autoantigen (GAD65 [glutamic acid decarboxylase]) may result from activation of a Th2 subset of T-cells that produce the cytokines IL-4 and IL-10 and consequently downregulate the Th1-cell–mediated autoimmune response. The clinical implication of these findings is that the autoimmune response leading to islet β-cell destruction and IDDM may be amenable to prevention or suppression by therapeutic interventions aimed at stimulating the host's own immunoregulatory mechanisms. *Diabetes* 43:613–621, 1994

The roles of cytokines in the pathogenesis of insulin-dependent diabetes mellitus (IDDM) have been the subject of recent reviews (1–4), which have focused on the cytotoxic effects of cytokines on islet β-cells and mechanisms that may mediate the inhibitory and destructive actions of cytokines on β-cells. However, contrary evidence is accumulating for diabetes-protective effects of cytokines in genetically diabetes-prone NOD mice and BB rats, animal models for human IDDM. Also, despite the well-recognized diabetes-preventative effects of immunosuppression, a variety of immunostimulatory agents have been discovered that prevent diabetes development in these animal models for IDDM. In this perspective article, I propose that these apparently conflicting observations regarding the diabetes-promoting versus diabetes-protective actions of cytokines, as well as the diabetes-protective effects of immunosuppression versus immunostimulation, can be accommodated and reconciled in the paradigm of autoimmune disease as a disorder of immunoregulation.

ENVIRONMENTAL INFLUENCES ON IDDM DEVELOPMENT
Environmental triggers of IDDM: the traditional view. Both genetic and environmental factors are involved in the pathogenesis of IDDM. The traditional concept is that environmental factors such as microbial agents and chemicals act as triggers of an autoimmune response against pancreatic islet β-cells in a genetically diabetes-prone phenotype (5,6). Indeed, certain viruses have been implicated in the induction of IDDM in humans, notably rubella (7), coxsackie (8,9), and cytomegalovirus (10,11). However, the evidence that human IDDM results from viral infection that affects islet β-cells directly or indirectly through the immune system is incomplete and inconclusive. There is some evidence for a viral etiology of IDDM in animal models with spontaneous autoimmune diabetes resembling human IDDM. Thus, a common rat parvovirus, Kilham's rat virus, has been identified as a viral trigger of IDDM in the BB rat (12). This microbial agent appears to promote diabetes by acting on cells of the immune system rather than on islet β-cells, and the investigators propose that the virus acts on effector or regulatory T-cells to promote somehow an autoimmune response against islet β-cells (12). In the NOD mouse, retrovirus-like particles have been identified in islet β-cells (13,14), and

differences have been reported between the expression of a retroviral gene in β-cells of diabetes-susceptible NOD mice and those of a control diabetes-resistant mouse strain (15). However, the possible relation of β-cell retroviral gene expression to the pathogenesis of β-cell damage and IDDM remains to be determined. Therefore, present evidence for the traditional view that microbial agents might trigger IDDM, either in human subjects or in animal models, is presently inconclusive.

Nevertheless, recent observations in autoimmune diabetes-prone animal models have reinforced the concept that environmental factors can have a profound influence on the expression of IDDM. Perhaps unexpected, however, is the recent strong evidence that exposure to microbial agents can prevent the development of IDDM.

Microbial agents can prevent IDDM: recent observations. Whereas evidence for induction of IDDM by environmental agents is inconclusive, recent observations have revealed that the environmental influence of microbial agents on IDDM can be protective against disease development (16). Thus, diabetes-prone NOD mice (16) and BB rats (17,18) raised in strictly pathogen-free environments manifest increased diabetes incidence, and viral infections in these animal colonies are associated with decreased diabetes incidence. These findings suggest that deliberate viral infection of diabetes-prone animals might actually protect against IDDM development.

This protection was demonstrated by experimental infection with the lymphocytic choriomeningitis (LCM) virus, which prevented diabetes in NOD mice (19) and BB rats (20). Although LCM virus is lymphotropic, the protection in NOD mice was not a result of production of an immunodeficient state, because lymphocytes recovered from LCM virus-infected mice failed to release infectious virus. Rather, LCM virus-infected and diabetes-protected NOD mice adoptively transferred protection from diabetes into young NOD mice (19). These findings suggest that viral infection somehow perturbed the immune system so that immunoregulatory or suppressor cells were activated to circumvent or suppress the autoimmune response against islet β-cells (16). In addition to LCM virus infection (19), mouse hepatitis virus (21), encephalomyocarditis virus (22), and lactate dehydrogenase virus (23) infections have been reported to prevent diabetes development in NOD mice.

Not only viruses, but also bacterial and fungal extracts have been discovered to exert protective effects against diabetes development in NOD mice and BB rats. These diabetes-protective microbial agents include a streptococcal extract, OK432 (24,25); staphylococcal enterotoxins (26); a fungal polypeptide, LZ-8 (27); and certain mycobacterial preparations, such as a 65-kDa heat shock protein (hsp 65) of *Mycobacterium tuberculosis* (28), a complete Freund's adjuvant (CFA) comprised of killed *Mycobacterium tuberculosis* in an oil emulsion (29–35), and the bacillus Calmette-Guérin vaccine (BCG) of live *Mycobacterium bovis* (35–39). A common feature of these microbial preparations is their stimulatory action on the immune system. This characteristic has qualified many of these microbial preparations as immune adjuvants, which are used to boost immune responses to antigenic challenges. Furthermore, pan T-cell–stimulatory lectins, such as concanavalin A and phytohemagglutinin have long been known to suppress rejection of tissue grafts and autoimmunity (40–42); and concanavalin A has been re-

ported to prevent diabetes in NOD mice (43), an action we have confirmed in diabetes-prone BB rats (A.R. W.L. Suarez-Pinzon, unpublished observations). It appears paradoxical that stimulation of the immune system should protect against diabetes considering the well-documented protective effects of immunosuppressive therapies in this autoimmune disease (44,45). The answer to this paradox lies in appreciating that the immune system operates in a network that involves finely regulated balances between different types of immune responses. Therefore, activation of one or another of the components of the immune system does not always lead to an increased immune response; rather, the opposite may result.

The strong and reproducible action of CFA to prevent diabetes development in autoimmune diabetes-prone NOD mice and BB rats has provided a very useful paradigm to study the setting of immune responses in genetically diabetes-prone animals and how immunostimulatory procedures may prevent the autoimmune response.

IMMUNE RESPONSES: ROLES OF CYTOKINES
The immune response to an antigen. The initial event in an immune response is the uptake and processing of antigen by macrophages, dendritic cells, or B-cells, which are termed collectively as antigen-presenting cells (APCs) because they present processed antigens to T-cells in association with major histocompatibility complex (MHC) class I or class II molecules at the surface of the APC. T-cells with specific receptors that recognize the antigen, T-cell antigen receptors, bind to the antigen-MHC complex. T-cells that respond to antigens complexed with MHC class I molecules are of the CD8+ phenotype (the CD8 molecule on the T-cell binds to the MHC class I molecule on the APC), and T-cells that respond to antigens complexed to MHC class II molecules are of the CD4+ phenotype (the CD4 molecule on the T-cell binds to the MHC class II molecule on the APC). T-cells also bind, by other ligands, to accessory (or adhesion) molecules on APCs. T-cell binding to the antigen-MHC complex and to accessory molecules on APCs leads to activation of the T-cells. One property of activated T-cells is cytokine production.

Characteristics of cytokines. Cytokines are peptide molecules synthesized and secreted by activated lymphocytes (lymphokines), macrophages/monocytes (monokines), and cells outside the immune system (e.g., endothelial cells, bone marrow stromal cells, and fibroblasts). Cytokines are used mainly by immune system cells to communicate with each other and to control local and systemic events of immune and inflammatory responses. More than 30 immunologically active cytokines exist and are generally grouped as interleukins (ILs), interferons (IFNs), tumor necrosis factors (TNFs), and colony-stimulation factors (CSFs) (46). Both the production of cytokines by cells and the actions of cytokines on cells are complex. Thus, one cell may produce several different cytokines; a given cytokine may be produced by one or several different cell types; and a cytokine may act on one or more cell types. Also, cytokine actions are usually local, occurring between two cells that are conjugated to one another, on neighboring cells (paracrine), and on the cell that secretes the cytokine (autocrine). In some cases (notably, the macrophage-derived inflammatory cytokines IL-1, IL-6, and TNF-α), cytokines exert actions on distant organs

TABLE 1
Cytokines produced by Th1- and Th2-cell subsets of T-cells

Cytokine produced	Th1-cell subset		Th2-cell subset	
	Mouse	Human	Mouse	Human
IL-2	+	++	–	–
IFN-γ	++	++	–	–
TNF-β	++	++	–	–
TNF-α	++	++	+	+
GM-CSF	++	+	+	++
IL-3	++	+	++	++
IL-4	–	–	++	++
IL-5	–	–	++	++
IL-10	–	+	++	++

Values for mouse Th1- and Th2-cell subsets are proportions of mouse CD4+ T-cell clones producing a given cytokine (50). Values for human Th1- and Th2-cell subsets are proportions of human T-cell clones producing a given cytokine (47). ++, Large proportion; +, small proportion; –, none.

.(endocrine). Cytokine synthesis is regulated by the differentiation of cells into the various cytokine-secreting phenotypes and by the selective activation of different cell types to produce some or all of their characteristic set of cytokines. **T-cell subsets, cytokine profiles, and immune response regulation.** Antigen-activated T-cells are termed T-helper (Th) cells because they help to mediate both cellular and humoral (antibody) immune responses. Th-cells are generally identified as CD4+; however, CD8+ Th-cells also exist (47). At least two distinct Th-cell types, Th1 and Th2, have been described, both in mice (48–50) and in humans (47). Th1- and Th2-cells are distinguished by their distinct cytokine secretion patterns (Table 1). Th1-cells produce IL-2, IFN-γ, and TNF-β (lymphotoxin), whereas Th2-cells produce IL-4, IL-5, and IL-10. Other cytokines are produced by both Th1- and Th2-cell populations. Also, Th-cell phenotypes other than Th1 and Th2 exist and have other patterns of cytokine secretion.

The functional significance of Th1- and Th2-cell subsets is that their distinct patterns of cytokine secretion lead to strikingly different T-cell actions (47–50). Th1-cells and their cytokine products (IL-2, IFN-γ, and TNF-β) are the mediators in cell-mediated immunity, formerly termed delayed-type hypersensitivity. Th1-cell–derived IFN-γ and TNF-β activate vascular endothelial cells to recruit circulating leukocytes into the tissues at the local site of antigen challenge, and they activate macrophages to eliminate the antigen-bearing cell. In addition, Th1-cell–derived IL-2 and IFN-γ activate cytotoxic T-cells to kill target cells expressing the appropriate MHC-associated antigen and activate natural killer cells to

kill target cells in an MHC-independent fashion. Thus, Th1 cytokines activate cellular immune responses. In contrast, Th2 cytokines are much more effective stimulators of humoral immune responses, i.e., immunoglobulin (antibody) production, especially immunoglobulin E, by B-cells. Furthermore, responses of Th1- and Th2-cells are mutually inhibitory. Thus, the Th1 cytokine IFN-γ inhibits the production of Th2 cytokines; these (IL-4 and IL-10), in turn, inhibit Th1 cytokine production.

Among signals that may orient the immune response in the direction of either a Th1- or a Th2-cell response, the macrophage-derived cytokines, IL-10 and IL-12, have been discovered to play important roles (51–53). IL-12 is a potent stimulant of Th1-cells and cytokines, notably IFN-γ. Thus, IL-12 can initiate cell-mediated immunity (54). In contrast, IL-10 (derived from macrophages or Th2-cells) exerts anti-inflammatory effects by inhibiting production of IL-12 and other pro-inflammatory macrophage cytokines (IL-1, IL-6, IL-8, TNF-α) by increasing macrophage production of IL-1 receptor antagonist and inhibiting the generation of oxygen and nitrogen free radicals by macrophages. In addition, IL-10 may favor Th2- over Th1-cell differentiation and function by inhibiting expression of MHC class II molecules and the B7 accessory molecule on macrophages, a major co-stimulator of T-cells (55). The combination of IL-4 and IL-10 is particularly effective in inhibiting Th1 effector function (cell-mediated immunity) in vivo (56).

It is evident, then, that activation of either Th1- or Th2-cells will result in either cellular or humoral immune responses, respectively. Protective responses to pathogens are dependent on activation of the appropriate Th subset accompanied by its characteristic set of immune effector functions. For example, human Th1-cells develop in response to intracellular bacteria and viruses, whereas Th2-cells develop in response to allergens and helminth components (47–50). Th1- and Th2-cells play different roles not only in protection against exogenous offending agents but also in immunopathology. Th1-cells are involved in contact dermatitis, organ-specific autoimmunity, and allograft rejection, whereas Th2-cells are responsible for initiation of the allergic cascade (47–50).

Evidence is accumulating that the islet β-cell–directed autoimmune response in IDDM may be a Th1 response, and that prevention of IDDM by immunostimulatory procedures may result from activation of an opposing Th2 response.

CYTOKINES IMPLICATED IN IDDM PATHOGENESIS
Studies over the last decade have examined through several different approaches the possible involvement of cytokines

TABLE 2
Cytokines implicated in IDDM pathogenesis

Cytokine action/production	IL-1	IL-2	IL-4	IL-10	TNF-α/-β	IFN-α	IFN-γ
Inhibits insulin secretion and may destroy β-cells in vitro	Yes	No	No	No	Yes	No	Yes
Present in insulitis lesion	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Transgenic β-cell expression leads to diabetes in normal mice	ND	No	ND	No	No	Yes	Yes
Anti-cytokine antibody prevents diabetes	ND	ND	ND	ND	Opposite	Yes	Yes
Administration of cytokine prevents diabetes	Yes	Yes	Yes	Yes	Yes	No	No
Cytokine production is decreased in diabetes-prone	Yes	Yes	Yes	ND	Yes	ND	Opposite

ND, not determined.

in the autoimmune pathogenesis of IDDM and in islet β-cell damage (Table 2). Studies in vitro have demonstrated that certain cytokines (IL-1, TNF-α, TNF-β, IFN-γ) can be directly cytotoxic to islet β-cells, inhibiting insulin secretion, and that, usually in combination, these cytokines can injure and destroy β-cells (1–4). Because these (and other) cytokines have been found to be expressed in the pancreatic insulitis lesion of NOD mice (57–60) and BB rats (61,62), IL-1, TNF-α, TNF-β, and IFN-γ may qualify as mediators of β-cell damage in IDDM. Also, IFN-γ has been detected in lymphocytes infiltrating islets of human subjects with recent-onset IDDM (63). Further evidence for IFN-γ being a β-cell cytotoxic cytokine in IDDM comes from the findings that transgenic expression of IFN-γ by β-cells in normal mice leads to an autoimmune, lymphocyte-dependent infiltration of the islets by mononuclear cells (insulitis), β-cell destruction, and IDDM (64,65). In addition, monoclonal antibodies to IFN-γ protect against diabetes development in NOD mice (57,66) and BB rats (67). Interestingly, IFN-α has been detected in β-cells of human subjects with recent-onset IDDM (68). Also, islet β-cell transgenic expression of IFN-α elicits an immune-mediated destruction of islet β-cells, and anti-IFN-α antibody prevents this β-cell damage and IDDM (69). However, IFN-α is a product of many cells that are virally infected or otherwise stressed, and this cytokine may recruit immune system cells (and their cytokines, e.g., IFN-γ) to damage the IFN-α–producing islet β-cells (69).

Studies involving the administration of cytokines to diabetes-prone NOD mice and BB rats in vivo have revealed that several cytokines can prevent diabetes development, including IL-2 (70,71), IL-4 (72), and IL-10 (73). Even some of the cytokines that are cytotoxic to β-cells in vitro (IL-1, TNF-α, and TNF-β) can prevent diabetes development in NOD mice and BB rats (74–79). Because deficiencies in the endogenous production of IL-1 (80), IL-2 (72,80), IL-4 (72), TNF-α (75,76,81), and TNF-β (79) have been reported in diabetes-prone NOD mice and/or BB rats, the diabetes-protective effects of chronic administration of these cytokines may represent corrections of immunoregulatory deficits in the diabetes-prone animals. However, systemic cytokine administration also may act indirectly on the immune system. For example, IL-1 and TNF can stimulate the hypothalamic-pituitary axis, leading to secretion of adrenocorticotropic hormone and, consequently, adrenal corticosteroids, which suppress inflammatory cells and cytokines (82).

Taken together, these studies (Table 2) indicate that the roles of certain cytokines in IDDM pathogenesis are uncertain; e.g., IL-1 and TNF may be cytotoxic to islet β-cells in the islet microenvironment (studies in vitro), but may prevent an islet β-cell–directed autoimmune response by acting on immunological or possibly neuro-endocrine cells (studies in vivo). On the other hand, the actions of other cytokines are more consistent: e.g., IFN-α and IFN-γ appear to have only diabetes-promoting roles, and IL-2, IL-4, and IL-10 appear to be diabetes-protective.

AUTOIMMUNE DIABETES: A TH1-CELL–MEDIATED IMMUNE PROCESS

Abundant evidence now suggests that autoreactive T-cells are present in the normal immune system but prevented from expressing their autoreactive potential by other regulatory (suppressor) T-cells (83). There is also good evidence for endogenous regulatory T-cells that oppose the emergence of

autoimmune diabetes in both NOD mice and BB rats (44). The opposing actions of autoreactive and regulatory T-cells are mediated by their respective cytokine products (47–50); and direct evidence for the operation of such a cytokine immunoregulatory balance in the avoidance of autoimmune diabetes has been provided recently (84). Diabetes was induced in a nonautoimmune rat strain by rendering the animals relatively T-cell–deficient using a protocol of adult thymectomy and sublethal γ-irradiation. Then, insulitis and diabetes were prevented in these rats by injection of a particular CD4+ T-cell subset that is isolated from healthy syngeneic donors and produces IL-4 and IL-2 but not IFN-γ (84). These findings are in accord with reports that IL-2 (72,80) and IL-4 (72) production are decreased in NOD mice and that administration of IL-2 (70,71) and IL-4 (72) can prevent insulitis and diabetes development. In addition, IL-10 administration recently has been found to decrease significantly insulitis severity and diabetes incidence in NOD mice (73). IL-4 and IL-10 are cytokine products of Th2-cells and inhibit cell-mediated immune responses, partly by downregulation of IFN-γ production by Th1-cells (51–52). Given the importance of IFN-γ as a mediator of islet β-cell destruction in vitro and of insulitis and diabetes in vivo (Table 2), it follows that IFN-γ–producing Th1-cells may contribute to the immune insulitis process that mediates islet β-cell destruction. Furthermore, the protective effects against insulitis and diabetes of IL-4 (72), IL-4–producing CD4+ T-cells (84), and IL-10 (73) suggest that Th2-cells producing IL-4 and IL-10 may be the T-cell subset responsible for preventing the autoimmune response, possibly by suppressing the IFN-γ–producing Th1 subset. Therefore, the concept arises that the autoimmune response in IDDM involves some disturbance(s) in immunoregulatory circuits that leads to a dominance of Th1 over Th2 T-cell subset function and cytokine production.

This concept that an immunoregulatory defect is associated with the autoimmune response to islet β-cells in IDDM posits that certain β-cell antigen(s) are processed by macrophages or other APCs and presented together with MHC class II molecules on the surface of the APC, resulting in the delivery of an immunogenic signal(s) that involves activation of CD4+ Th1-cells and suppression of CD4+ Th2-cells and their respective cytokines (Fig. 1). Although the molecular nature of the putative immunogenic signals involved in the autoimmune response of IDDM is unknown, these signals likely relate to mechanisms involved in recognition of MHC-antigen complex by T-cells. Thus, the immunogenicity of a β-cell protein may depend on the peptide fragment derived from processing by the APC (85), the amino acid sequences of the MHC class II molecules that bind and present the β-cell peptide (antigen), and the precursor frequency of autoreactive T-cells with T-cell receptors to match the β-cell antigen-MHC complex (86). In addition to the MHC-antigen complex interaction with T-cell receptors, co-stimulation of T-cells by interaction with APC accessory (or adhesion) molecules is necessary for full T-cell activation (87). For example, transfer of diabetes in NOD mice is prevented by blockade of an adhesion-promoting receptor on macrophages (88). Also, pancreatic islet grafts survived xenogeneic transplantation (from rats to streptozocin-induced diabetic mice) when the mice were treated with an immunoligand that binds the B7 adhesion molecule on APCs (89). Finally,
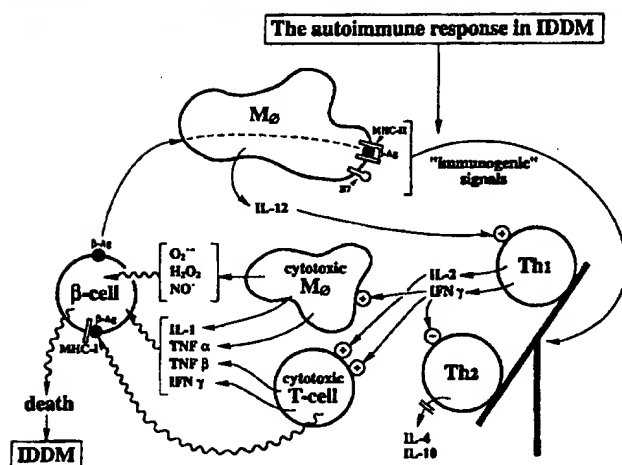
FIG. 1. A scheme illustrating the immune system cells that may be involved in the autoimmune response leading to destruction of pancreatic islet β-cells and IDDM. The concept illustrated posits that certain β-cell protein(s) act as autoantigens (β-Ag) after being processed by macrophages (Mφ) or other antigen-presenting cells and presented in a complex with MHC class II molecules on the surface of the Mφ. The β-Ag-MHC II complex, accessory molecules on the Mφ (e.g., the B7 molecule), and perhaps other signals together may comprise immunogenic signals that activate T-cells, predominantly of the Th1 subset. Also, Mφ-derived IL-12 activates Th1-cells. The antigen-activated Th1-cells produce IL-2 and IFN-γ, which inhibit Th2-cell production of IL-4 and IL-10. Also, IL-2 and IFN-γ activate Mφ and cytotoxic T-cells to kill islet β-cells by a variety of mechanisms, including oxygen free radicals (O₂⁻ and H₂O₂), NO⁻, cytokines (IL-1, TNF-α, TNF-β, IFN-γ), and cytotoxic T-cells that interact with a β-cell autoantigen-MHC class I complex on the β-cell.
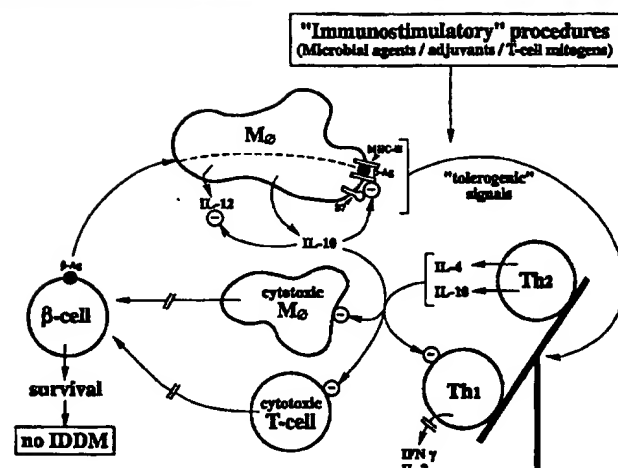
FIG. 2. A scheme illustrating possible mechanisms by which different immunostimulatory procedures (microbial agents, adjuvants, T-cell mitogens) may provide "tolerogenic" signals that would substitute for the "immunogenic" signals mediating the autoimmune response in IDDM (Fig. 1). Thus, islet β-cell autoantigen (β-Ag) processing and presentation by macrophages (Mφ) would deliver "tolerogenic" signals that activate a Th2 subset of T-cells producing IL-4 and IL-10 that downregulate Th1-cells and IFN-γ and IL-2 production. Also, IL-10 production by Mφ may be upregulated by these immunostimulatory procedures, with consequent downregulation of β-Ag presentation and reinforcement of a "tolerogenic signal" for recognition of β-Ag as self by the immune system. In addition, IL-10 inhibits production of IL-12, a Th1-cell activator. The combination of upregulated IL-4 and IL-10 production and downregulated IFN-γ and IL-2 production would inhibit cytotoxic Mφ and T-cell functions and thereby prevent β-cell damage and IDDM.

the direction taken by the T-cell response, in terms of Th phenotype, is largely regulated by cytokines. Thus, naive T-cells are not precommitted to any particular Th phenotype; the Th phenotype varies with the cytokines in the microenvironment. The presence of IL-12, a macrophage and B-cell product, favors Th1-cell differentiation, and anti-IL-12 antiserum blocks expression of the Th1 phenotype (53,54). Alternatively, IL-4, a Th2 and possibly a mast cell product (54), favors Th2-cell differentiation, and anti-IL-4 monoclonal antibody promotes expression of a Th1 phenotype (90,91).

The results of CD4+ Th1-cell activation are induction of IL-2 and IFN-γ production; inhibition of Th2 cytokine production; and activation of macrophages, cytotoxic T-cells, and natural killer cells. These activated effector cells may be cytotoxic to islet β-cells through a variety of mechanisms (Fig. 1), including oxygen free radicals (O₂⁻ and H₂O₂), nitric oxide (NO⁻), cytokines (IL-1, TNF-α, TNF-β, IFN-γ) that may be directly cytotoxic to islet β-cells, cytotoxic (CD8+) T-cells that interact with an islet β-cell autoantigen-MHC class I complex on the β-cells, and natural killer cells that may damage β-cells without MHC restriction. The predominance of one or another of these potential immune/inflammatory mediators of islet β-cell destruction is the subject of continuing studies in animal models (1–4,92–101) and in human subjects with IDDM (102–105).

In addition to macrophages and T-cells, other cellular elements in and around the islet (not shown in Fig. 1) are likely participants in the insulitis lesion. For example, vascular endothelial cells may contribute cytokines (IL-1 and IL-6) and may respond to inflammatory cytokines (IL-1, TNF, and IFN-γ) by expressing adhesion molecules to circulating leukocytes (106). This response would permit migration of

macrophages and lymphocytes from the circulation into the islet. Also, endothelial cells may respond to inflammatory cytokines by expressing MHC class II molecules (107), which could allow endothelial cells to act as APCs and possibly present β-cell autoantigen(s) to T-cells. Thus, intra- and peri-islet vascular endothelial cells could participate actively in amplifying the β-cell–directed autoimmune process (108).

IMMUNOSTIMULATORY PROCEDURES PREVENT IDDM: CORRECTION OF A CYTOKINE BALANCE?

The concept has been presented above that the autoimmune response in IDDM involves some disturbance(s) in immunoregulatory circuits that is manifested as dominance of Th1 over Th2 T-cell subset function and cytokine production (Fig. 1). A corollary of this proposition is that measures leading to reversal of this Th subset balance, with Th2-cells/cytokines dominating over Th1-cells/cytokines, should block the autoimmune response and prevent IDDM. Evidence is building to support this possibility. Thus, a variety of immunostimulatory procedures that include certain microbial agents and extracts, immune adjuvants, and T-cell mitogens (19–39,43) recently have been discovered to prevent the development of insulitis, β-cell destruction, and IDDM in genetically diabetes-prone animals (see Microbial agents.) These procedures may provide tolerogenic signals that substitute for the immunogenic signals operant in autoimmune IDDM and thereby reset the Th subset balance so that Th2-cells/cytokines now dominate over Th1-cells/cytokines (Fig. 2).

Autoimmunity is generally viewed as a failure of the immune system to develop tolerance or nonreactivity to self molecules (potential antigens). Tolerance of the immune

system to self may be established by different mechanisms, including clonal deletion (elimination) of autoreactive T-cells, clonal anergy (paralysis) of autoreactive T-cells, and suppression of autoreactive T-cells by other cells or products of the immune system, e.g., by nonspecific suppressor cells, antigen-specific T regulatory (suppressor) cells, and immunoregulatory cytokines. One or more of these mechanisms of T-cell tolerance (except clonal deletion) may be involved in the actions of microbial agents and adjuvants that lead to the prevention of autoimmune diabetes. Certainly, these immunostimulatory procedures prevent diabetes development in genetically diabetes-prone NOD mice and BB rats (19–39,43) without structural changes or complete remodelling of the immune system—unlike procedures that involve bone marrow, thymic, or lymphoid cell replacement or deletion, e.g., anti-lymphocyte serum, cyclosporine, monoclonal antibodies to T-cells, silica, and anti-macrophage antibodies (44).

Evidence has been presented for CFA-induced protection against diabetes development in NOD mice and BB rats in association with increases or induction of antigen-nonspecific or natural suppressor (NS) cells (29,31,34). NS cells are generally considered to be large granular lymphocytes belonging to the T-cell lineage but lacking mature T-cell markers; i.e., NS cells are CD4−CD8− (109). How NS cells exert their suppression of T- and B-cell functions is not known. However, the suppression may be effected by one or more factors and cytokines produced by NS cells (110,111). Macrophages with nonspecific suppressor activity have also been reported to be induced by the immune adjuvant BCG in association with protection from diabetes in NOD mice (37,38).

Most studies, however, have identified T regulatory cells and cytokines as mediators of the diabetes-protective effects of immune adjuvants (33,35). T-cells induced after CFA treatment of NOD mice can prevent both the inductive and effector phases of the autoimmune response that leads to islet β-cell destruction and IDDM (33,35). Thus, lymph node or splenic cells from CFA-treated NOD mice transferred protection from diabetes in young NOD mice; also, adoptive transfer of spleen cells from CFA-treated NOD mice, together with spleen cells from acutely diabetic NOD mice, delayed disease induction in irradiated recipient NOD mice (35). Depletion of the Thy1.2+ (total T) cells or the CD4+ (Th) cells from the CFA-treated NOD donor splenic cells abrogated the protective effects of these cells, indicating that the CFA-induced protective cells were CD4+ Th cells (35). In addition, CFA-treated old NOD mice were resistant to passive transfer of disease by spleen cells from acutely diabetic NOD mice; however, diabetes could be induced in the CFA-protected mice by cyclophosphamide treatment, which suggests that T regulatory cells (presumably deleted by cyclophosphamide) accounted for the protective effects of CFA against the autoimmune response. Similarly, protection against diabetes in NOD mice injected with staphylococcal enterotoxins, called superantigens because they stimulate a large fraction of T-cells, was attributed to activation of CD4+ T suppressor cells (26).

The ability of CFA to inhibit the effector phase of diabetes in the studies above (35) confirmed an earlier report that treatment of already diabetic NOD mice with CFA at the time of syngeneic islet transplantation prevented islet β-cell destruction and disease recurrence (32). In these experiments, monocytic and lymphocytic cells still accumulated around the transplanted islets (peri-insulitis) in the CFA-treated NOD mice, but these cells did not invade the islet, and insulin-containing β-cells remained intact even 200 days after islet transplantation (32). These findings suggest that autoreactive T-cells exist in the CFA-treated NOD mice but cannot function as effectors of β-cell destruction. Similar results were reported in another study where autoreactive T-cells were considered to be dormant in the CFA-treated NOD mice (33).

Taken together, these studies suggest that the mycobacterial immune adjuvants CFA and BCG (and possibly other microbial agents and T-cell mitogens) may deliver tolerogenic signals, i.e., activate regulatory (suppressor) T-cells that would render islet β-cell autoreactive T-cells nonresponsive. Furthermore, the regulatory T-cells are of the CD4+ Th type and may belong to the Th2 subset that produces IL-4 and IL-10, as suggested by the following studies.

Protection against β-cell destructive insulitis and diabetes in NOD mice, provided by injecting the mice with CFA, was reported to be associated with a relative increase in IL-4–producing T-cells and a decrease in IFN-γ–producing T-cells recovered from sentinel syngeneic islet grafts placed under the renal capsule in NOD mice (112). In addition, by using a polymerase chain reaction assay to measure cytokine mRNA expression in tissues, we have found that IL-10 mRNA expression is significantly increased and expression of IL-2 and IFN-γ mRNAs is significantly decreased in syngeneic islet grafts of CFA-injected NOD mice compared with saline-injected NOD mice (112a). Therefore, we concluded that the β-cell destructive infiltrate in syngeneic islet grafts transplanted into diabetic NOD mice contained IL-2 and IFN-γ–producing Th1-cells and that CFA treatment of the diabetic NOD mice at the time of islet transplantation induced IL-10–producing cells that downregulated the Th1-cells, converting a β-cell destructive islet infiltrate into a nondestructive one and thereby preventing islet graft rejection and diabetes recurrence. This interpretation is supported by the finding that administration of IL-10 significantly prolonged survival of syngeneic islet grafts in diabetic NOD mice (112b). Also, another study reports that IL-10 administration can significantly decrease insulitis severity and the incidence of spontaneous diabetes in NOD mice (73). These effects of IL-10 are in accord with the known actions of this cytokine to downregulate inflammatory responses mediated by monocytes/macrophages and their cytokine products, as well as to downregulate cell-mediated immune responses triggered by Th1-cells and their cytokine products (51–55). Also, our finding of increased IL-10 mRNA expression in nondestructive islet infiltrates (CFA-protected syngeneic islet grafts in NOD mice, 112a) is in accord with a report that IL-10 mRNA expression in the central nervous system of mice with experimental autoimmune encephalitis (a model for multiple sclerosis) correlates with recovery from disease (113). Interestingly, transgenic expression of IL-10 by islet β-cells in mice leads to pronounced vascular endothelial cell changes and leukocyte extravasation into the pancreas without infiltration of cells into the islets, β-cell destruction, or diabetes (114).

Taken together, these studies suggest that immunostimulatory procedures, such as certain microbial agents and immune adjuvants, may stimulate the production of regulatory cytokines, such as IL-4 (by Th2 and/or mast cells) and IL-10 (by Th2 and/or macrophages). These cytokines could

contribute to tolerogenic signals, i.e., signals for the immune system to recognize islet β-cell potential autoantigen(s) as self. Thus, tolerogenic signals would substitute for immunogenic signals that direct an autoimmune response against islet β-cells. These tolerogenic signals would favor T-cell differentiation along a Th2 pathway, downregulate Th1-cells and cytokines (IFN-γ, IL-2, TNF-β), inhibit cytotoxic macrophage and T-cell functions, and consequently preserve islet β-cells and avoid IDDM (Fig. 2).

FUTURE PROSPECTS: CLINICAL CONSIDERATIONS

The clinical hope from the observations that certain immunostimulatory procedures prevent autoimmune diabetes development in genetically diabetes-prone animals is that clinically safe means of immune stimulation may be similarly effective in preventing IDDM in human subjects at risk for this disease. Immunostimulatory agents that have a broad spectrum of immune stimulation, affecting macrophages and ·T-cells (such as the immune adjuvant BCG) and polyclonal T-cell activators (such as microbial superantigens and lectins) may not be optimal for clinical trials because of possible undesirable side effects from generalized immunostimulation.

However, recent findings demonstrate that more selective immunostimulation may be at hand. Thus, administration of the peptide GAD65 (glutamic acid decarboxylase), an islet β-cell autoantigen, can prevent autoimmune diabetes development in NOD mice, and this prevention is associated with the induction of specific tolerance to this peptide (115–117). Moreover, GAD-responsive T-cells from diabetes-prone NOD mice were characterized as Th1, IFN-γ–producing (116), whereas IFN-γ production in antigen-stimulated spleen cell cultures from GAD65-tolerant (and diabetes-protected) NOD mice was reduced significantly, indicating that tolerance may result from suppression of GAD65-responsive Th1-cells (117). Because this effect was not accompanied by a corresponding reduction of the humoral (antibody) response to GAD and other IDDM autoantigens, a GAD65 induction of Th2-cells with suppression of Th1-cells was suggested (117). These findings are directly relevant to the observation that in humans there is an inverse relation between humoral (Th2-cell–mediated) and cellular (Th1-cell–mediated) autoimmunity to GAD in subjects at risk for IDDM (118); also, a strong humoral response to GAD correlates with a slow progression to IDDM (118,119).

Therefore, the paradigm of autoimmune diabetes as a Th1-cell–mediated immune response involving IL-2 and IFN-γ–induced activation of cytotoxic macrophage and T-cell killing of islet β-cells, based on recent evidence in animal models, may also apply to human IDDM. Conclusive evidence for similar involvement of Th1-cells and cytokines in the pathogenesis of human IDDM, however, remains to be obtained. Some evidence exists for other organ-specific autoimmune disorders, including Hashimoto's thyroiditis (120) and progressive multiple sclerosis (121).

In summary, nonspecific stimulation of the immune system, by administration of microbial extracts and adjuvants, as well as specific immune stimulation by administration of an islet β-cell autoantigen, GAD65, can prevent autoimmune diabetes development in genetically diabetes-prone NOD mice. Both nonspecific and specific immune stimulations appear to prevent diabetes by downregulating a Th1 subset

of T-cells and their cytokine products (IFN-γ and IL-2) and by upregulating a Th2 subset of T-cells and their cytokines (IL-4 and IL-10). Although it remains to be demonstrated that human IDDM involves similar Th1-cell and cytokine-mediated autoimmune processes, these findings in NOD mice provide a rationale to consider immune modulation therapies involving immunostimulation in attempts to prevent IDDM in human subjects at risk for this disease. In addition, identification of the cytokine mediators and suppressors of the islet β-cell–directed autoimmune response in animal models with IDDM suggests the use of these peptides (or their antagonists) for therapeutic intervention in human IDDM prevention.

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